The Role of Transitional B cells in Kidney Transplantation Tolerance

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The Role of Transitional B cells in Kidney Transplantation Tolerance

A thesis submitted to the School of Medicine at King's College London for the degree of Doctor of Philosophy

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Declaration

This thesis is a presentation of my original research work. Wherever contributions of others involved was indicated with due reference to the literature, and acknowledgement of collaborative research and discussions.

ESTEFANÍA ANDREA NOVA LAMPERTI

Dedicated to my beloved grandfather

MIGUEL NOVA A.

(1933-2011)

Abstract

Previous studies aimed at identifying biomarkers of tolerance in kidney transplant patients have revealed an expansion of peripheral blood B cells and over-expression of B cell-related genes. In humans, Memory, Naïve and Transitional B cells are the main B cell subsets found in circulation, and it is has been shown that the Transitional subset, defined by its regulatory properties, is expanded in tolerant recipients compared to non-tolerant kidney transplant patients. However, the role this population plays in kidney transplantation tolerance remains unclear. Here, we report three different mechanisms to explain the contribution of B cells in transplantation tolerance.

Kidney transplant patients were divided into five groups; tolerant, stable, monotherapy, patients who lost tolerance and chronic rejector. In addition, this study also included a group of age/gender-matched healthy volunteers. B cells from each group of patients were tested for antigen presentation, antibody production, cytokine production, and co-stimulatory function.

B cells from tolerant patients produced higher levels of IL-10 and lower levels of TNF-α than B cells from chronic rejector after CD40 and CpG activation. Moreover, B lymphocytes from tolerant patients also exhibited a failure in the BCR signalling pathway, suggesting a certain degree of anergy or responsiveness by these cells. Donor-specific assays revealed that B cells from tolerant patients were inefficient to recognise donor-antigens, compared to B cells from chronic rejector. This impairment prevented the triggering of the Th1 response by recipient $CD4^+$ T cells and donor-specific antibody production by Plasma cells. Finally, Transitional B cells were the lowest $CD4^+$ T cell-activating cells, compared to Naïve and Memory B cells. This reduced $CD4^+$ T cell activation was due to low cell viability, reduced CD86 expression and high IL-10 production.

In conclusion, these data suggest that B cells from kidney tolerant recipients contributed to maintaining organ acceptance and graft survival by donor-specific and non-specific regulatory properties exhibited by all their subsets, especially Transitional B cells.

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Standards annotations

In this thesis, a conventional annotation is used to differentiate genes (*italic*) and proteins (normal).

List of abbreviations

APC	Allophycocyanin
APCs	Antigen-Presenting Cells
BAFF	B-cell Activating Factor
BAFFr	B-cell Activating Factor Receptor
BCR	B cell Receptor
BD	Becton Dickinson
BLNK	B-cell Linker
BSA	Bovine Serum Albumin
BTK	Bruton's Tyrosine Kinase
CD	Cluster of Differentiation
CD40L	CD40 Ligand or CD154
CFSE	5-(and -6)-CarboxyFluorescein diacetate Succinimidyl Ester
CpG	Cytosine phosphodiester Guanine
CR	Chronic Rejector
CRT	Creatinine
CS&T	Cytometer Setup & Tracking Beads
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
Delta ERK-p	▲ERK-p
DMSO	Di-Methyl Sulfoxide
DCs	Dendritic cells
EDTA	Ethylene Di-amine Tetra Acetic Acid
ELISA	Enzyme-Linked ImmunoSorbet Assay
ELISpot	Enzyme-Linked ImmunoSorbent spot
ERK	Extracellular signal-Regulated Kinase
ERK-p	Extracellular signal-Regulated Kinase -phosphate

FCRL	Fc Receptor Like
FBS/FCS	Foetal Bovine Serum/Foetal Calf Serum
FITC	Fluorescein Iso Thio Cyanate
FO	Follicular B cells
GAMBIT	Genomic and Molecular Biomarker of Immunological Tolerance
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HC	Healthy Control
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
ICAM	Intercellular Adhesion Molecule
ITAM	Immunoreceptor-Tyrosine-based Activation Motif
IFN-γg	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IOT	Indices of Tolerance
LFA	Lymphocyte Function-Associated Antigen
LT	Lymphotoxin or TNF-β
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Antigens
mHC	Minor Histocompatibility Antigens
MMF	Mycophenolate Mofetil
Mon	Monotherapy
MS4A1	Membrane-Spanning 4-domains
MZ	Marginal Zone
MZP	Marginal Zone Precursor
NKs	Natural Killers
NKTs	Natural Killer T cells

PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECy	Phycoerythrin-Cy
PerCP	Peridinin-Chlorophyll-Protein complex
PMA	Phorbol 12-Myristate 13-Acetate
RPM	Revolutions per Minute
RPMI	Roswell Park Memorial Institute Medium
Sta	Stable
T-1	Transitional 1
T-2	Transitional 2
T-3	Transitional 3
TCR	T cell Receptor
TD	Thymus Dependent (TD)
TGF-βb	Transforming Growth Factor βeta
Th	T helper
TI	Thymus Independent
TLR	Toll-Like Receptor
TMB	Tetra Methyl Benzidine
TNF-α	Tumour Necrosis Factor alpha
Tol	Tolerant

1 Introduction

1.1 Kidney Transplantation

Transplantation is the medical treatment used to replace tissues that are no longer functional within humans. This treatment has undergone many advances since its discovery; types of transplant, organs used, antigen recognition and complementary medication are some of the aspects that have improved over time. A brief description documenting organ transplantation from its early times will be discussed below to contextualise the research project carried out in this thesis.

1.1.1 Brief History of Organ Transplantation

Documented reports of organ transplantation begins in 1869, when Swiss surgeon Jacques Louis Reverdin performed the first clinical experimental transplant between two genetically different individuals, using fresh human skin as a source for the allograft. Skin then became the first tissue used in human transplantation. Then, in 1905, a second tissue was introduced into this field. Austrian surgeon Eduard Zirm used a cornea from an 11-year-old boy to restore the sight of Alois Glogar, a labourer who had been blinded by accidentally burning his eyes with caustic lime.

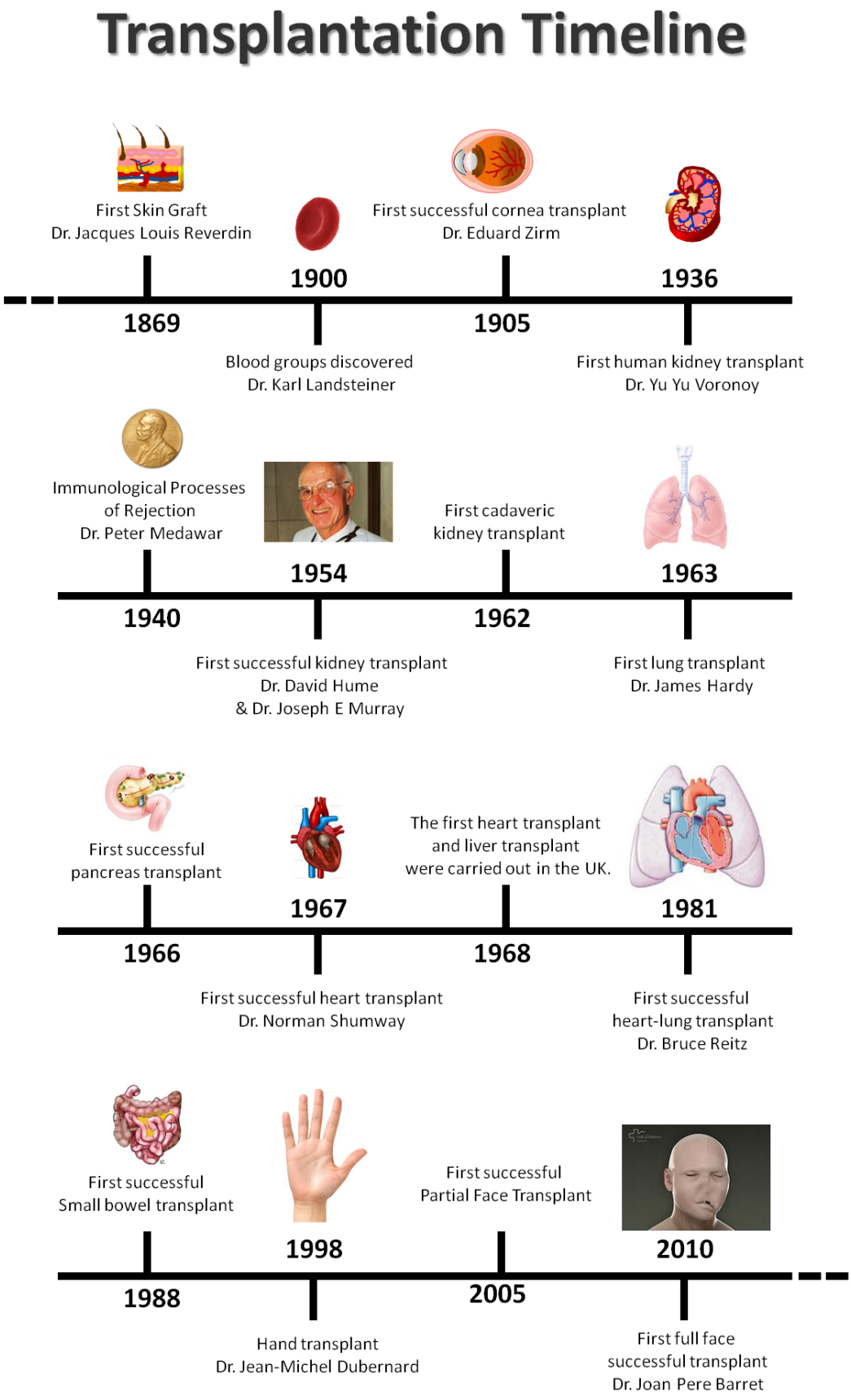
In the 1940s, UK researcher Peter Medawar studied the phenomenon of allograft rejection, and recognised that whereas autologous skin grafts were accepted, those given from one relative to another were rejected; he received the Nobel Prize in 1960 for demonstrating that graft rejection can be entirely prevented in mice and chickens if foreign cells from the future donor are introduced into the recipient during foetal or neonatal life (Terasaki 1991). In 1954, kidneys were used in human transplantation, with great success. Dr. Joseph E. Murray, who received the Nobel Prize for Medicine in 1990, achieved the first successful kidney transplant between identical twins in Boston, USA. Since then, the field of transplantation has seen a wealth of successful transplants using a variety of different tissues. For instance, in the early 1960s, transplants of lung, pancreas and liver were achieved, and in 1967, the first human heart transplant was performed by a South African heart surgeon named Christian Barnard at Groote Schuur Hospital in Cape Town. Christian Barnard replaced the diseased heart of dentist Louis

Washkansky with that of a young accident victim. Although immunosuppressive drugs prevented rejection, Washkansky died of pneumonia 18 days later.

The first heart transplant and liver transplant to be carried out in the UK occurred in 1968, and three years later, donor cards were introduced to help supply the need for organs. Over time, organ transplantation has become a regular treatment used in the USA, Europe and the rest of the world.

In 1978, the immunosuppressive drug cyclosporine was successfully introduced into organ transplantation, and was approved by the USA Food and Drug Administration (FDA) in 1983. In 1994, the FDA approved FK506 or tacrolimus, another fungal product. This drug exhibited the same mode of action as cyclosporine, but with a higher immunosuppressant effect.

Finally and more recently, hands, partial face and complete face transplants have been performed in France, China, the USA and Spain (Brent 1997; Epstein 2011); and it is expected that continued clinical, scientific and technological advances will lead to better outcomes and a larger variety of tissues for allotransplantation in the following years (wellcomecollection.org) (Picture 1).



Picture 1: **Transplantation Time Line**

1.1.2 Solid Organ Transplantation in Europe and the UK

Solid organ transplantation is the treatment of choice for individuals with severe organ failure. Once the organ has been replaced, stable graft function and patient survival are the best indicators for the success of the transplant. Survival rates of transplant patients have improved over the last few decades, indicating that the treatment is evolving and improving. Despite these successes, the limited number of organs available for donation remains the main obstacle in transplantation.

Between 2006 and 2007, over 3,000 patients in the UK received a transplant, but another 1,000 died whilst waiting, or after being removed from the waiting list because they had become too ill (Organ Donation 2008). The UK has one of the lowest organ donation rates in the developed world; the current active transplant waiting list stands at 7,235 and is rising by approximately 8% each year (Organ Donation 2008). In 1995, the UK and Ireland donor rate was 15,8 per million populations (pmp), and was comparable with the 15,1 pmp rate reported by the Eurotransplant (the Netherlands, Belgium, Germany, Luxembourg, Austria, Slovenia and Croatia), and the 15,5 pmp rate reported in France. More recently, the UK rate has dropped to 12,9 pmp, while Spain has achieved 35,5 pmp (Organ Donation 2008). This wide gap demonstrates the necessity of donation campaigns and better organ clinical care in the UK.

Finally, around 100,000 solid organ transplants are performed per year worldwide; 68,250 kidney, 19,850 liver, 5,179 heart, 3,245 lung, and 2,797 pancreas (Matesanz *et al*. 2009). The kidney is by far the main organ used in transplantation, mainly because a single kidney has enough functionality to allow the donor and recipient to survive with a good quality of life. In the following sections, kidney transplantation will be reviewed in more detail as the samples used in this thesis were obtained from kidney transplant recipients.

1.1.3 Kidney Transplantation Origin

In 1954, Joseph Murray and colleagues performed the first successful kidney transplant at Boston's Peter Bent Brigham Hospital, in the USA. The team had previously carried out a series of human kidney grafts with partial success, maintaining stable organ function only for a couple of days or even months. In 1954, the surgeons transplanted a kidney from 23-year-old Ronald Herrick into his twin brother Richard.

As the donor and recipient were genetically identical, the procedure was successful. Dr. Murray received the Nobel Prize for Medicine in 1990.

A number of important advances in the field of kidney transplantation have been made in the past years regarding individual matching, immunosuppressive medication, origin of the organ, and quality of life of the recipient. The following paragraphs will introduce the immune responses operating in kidney transplantation, the effect of immunosuppressive drugs used to maintain stable graft function and the different outcomes displayed by recipients, especially the interesting development of spontaneous transplantation tolerance.

1.1.4 Kidney Transplantation

Transplantation is the gold-standard treatment for end-stage diseases in solid organs. In the context of end-stage kidney disease, transplantation is superior to other forms of renal replacement therapy (RRT), as it improves both morbidity (quality of life) and mortality (Wolfe *et al*. 1999; Magee *et al*. 2004).

Unlike other types of transplants, a living person can donate a kidney because it is possible to survive with one remaining organ; this type of donation is called living donation. Donation can also come from deceased donors, but their long-term survival is lower when compared to organs from a living donor (Hariharan *et al*. 2000).

Immediately after transplantation (independently of the type of donation), the allorecognition towards the foreign organ plus the damage caused by surgery, both trigger the activation of the cardiovascular and immune system. In order to control these responses from the surgery, immunosuppressive drugs have to be taken by transplant recipients for the rest of their life (Chinen *et al*. 2010). Unfortunately, immunosuppressant regimens elicit unpleasant adverse side effects such as increased risk of infection, cardiovascular disease and cancer (Chinen *et al*. 2010). Therefore, transplanted patients have to balance both effects for life, using these drugs at optimal concentrations to control their immune system with minimal side-effects (Womer *et al*. 2001).

1.1.5 Immunological effect of Kidney Transplantation

One of the main characteristics of the immune system is its ability to distinguish self from non-self antigens therefore, the activation of immune responses in a healthy individual should be triggered only by foreign antigens (Eremin *et al*. 2011). The final outcome of a kidney transplant will depend on a good control of the alloresponse in each particular patient.

The innate and the adaptive immune responses both play a critical role in rejection and are activated after transplantation. The innate response is induced by tissue stress through the interactions between damaged cells and pattern recognition receptors (PRRs) on neutrophils, monocytes, and dendritic cells (DCs). As a result of this activation, reactive oxygen species (ROS) and pro-inflammatory cytokines such as TNF-α are released (Murphy *et al*. 2012). The adaptive immune response begins with the recognition of certain donor-proteins as foreign or non-self antigens. One of the most important proteins in this recognition are the highly polymorphic class I and class II cell surface molecules encoded by the Major Histocompatibility Complex (MHC) genes (the Human Leukocyte Antigen (HLA) system in humans) (Snell 1951; Snell *et al*. 1951). Additionally, the ABO system and minor Histocompatibility (mH) antigens are also important antigens to consider in human transplantation.

1.1.6 Transplantation Antigens

MHC

The MHC was described in the mouse model in 1948 by George Snell and Peter Gorer. They designated individual histocompatibility loci using the letter *H* and a serial number; the first identified *H* locus was designated *H2* in recognition of the fact that it encoded antigen II (Snell 1948). Snell and colleagues also provided the first indication that *MHC* genes might be highly polymorphic. Several years later, the HLA system was recognised as the human version of MHC. The genes of the HLA locus are located on the short arm of chromosome six, and they encode two distinct classes of cell surface molecules: class I and class II (Duquesnoy *et al*. 1988).

Humans have three main highly polymorphic MHC class I genes, known as *HLA-A, HLA-B, and HLA-C*. These genes transcribe proteins which, in combination with the β 2-microglobulin (β 2M), form the class I molecules HLA-A, HLA-B, and HLA-C. There are six main MHC class II genes in humans: *HLA-DPA1, HLA-DPB1,*

HLA-DQA1, HLA-DQB1, HLA-DRA, and *HLA-DRB1*. These genes encode the class II molecules HLA-DR, -DP, and -DQ (Bach *et al*. 1967; Mehra *et al*. 2003; Eremin *et al*. 2011) (Picture 2).

Peptides presented by class I HLA molecules are derived from cytosolic proteins, such as viral pathogens and their gene products, whereas peptides presented by class II molecules are generated by breakdown of endosomal proteins. Class I molecules are expressed constitutively by most nucleated cells of the body, but class II molecules are restricted to cells that have the capability to process and present exogenous soluble and particulate antigens such as B lymphocytes, DCs, macrophages, and monocytes therefore, those cells are called antigen-presenting cells (APCs) (Halloran *et al*. 1986; Eremin *et al*. 2011; Murphy *et al*. 2012).

According to the latest data available from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/stats.html), a total of 3,296 HLA-A, -B, and -C α -chain alleles (expressed as 2,520 distinct proteins), have been identified. In practice, three of the HLA loci are usually typed and matched for kidney transplantation: HLA-A, HLA-B, and HLA-DR.

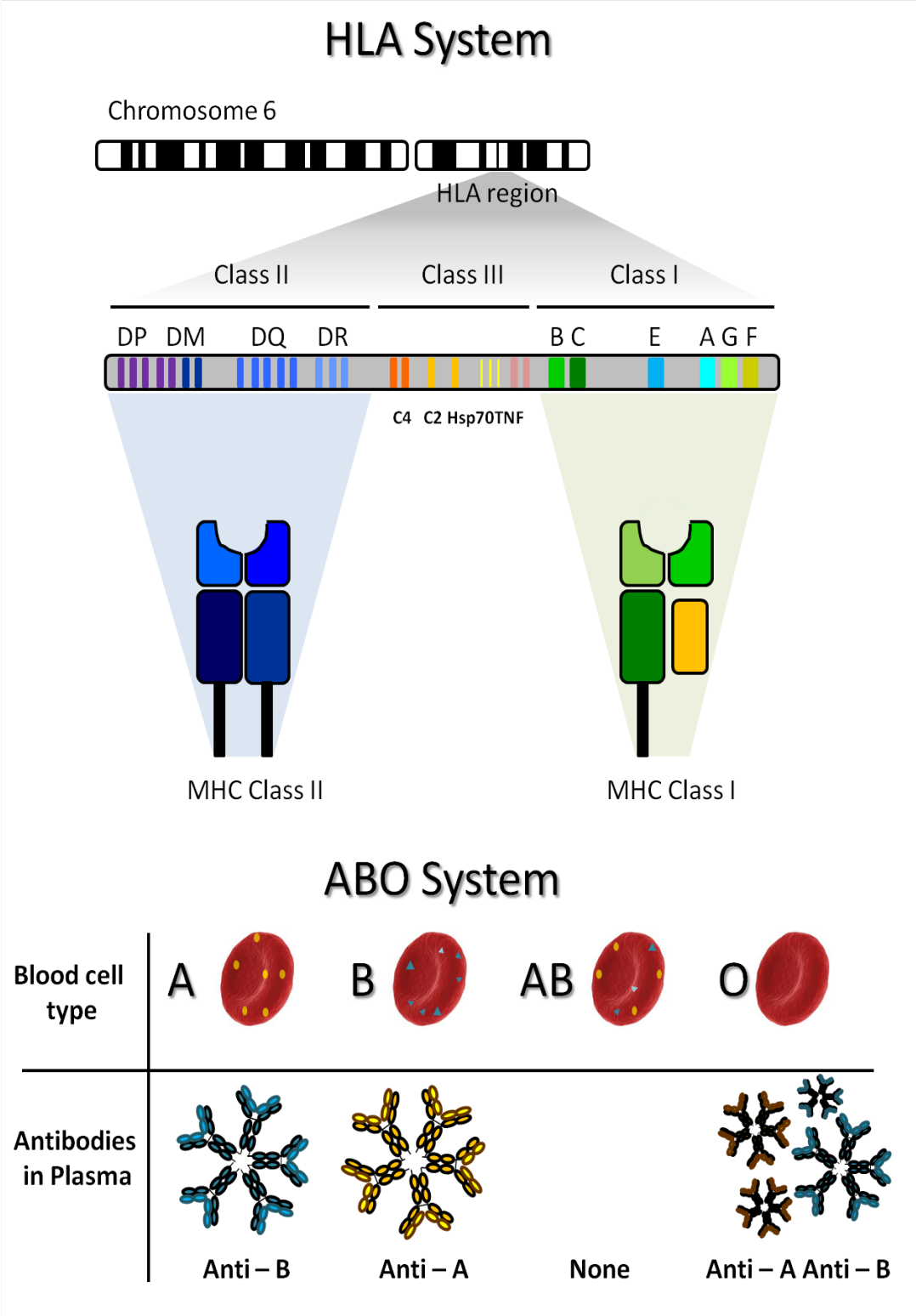
Given their high polymorphism and their ability to stimulate strong immune responses, it was recognised long ago that transplant outcomes were much improved when donor and recipient were well matched.

ABO

Erythrocytes, or red blood cells, express a number of protein–carbohydrate molecules that stimulate coagulation responses. The best studied are those stimulated by the H antigens. These antigens have three allelic forms: A antigens, B antigens, or unchanged H antigens designated as O (Picture 2). Every person possesses naturally-occurring IgM antibodies against their non-expressed A or B antigens, developed during the childhood in response to bacteria that express cross-reactive antigens in the gastrointestinal tract. ABO matching is a requirement for organ transplantation, although there are now effective plasmapheresis procedures for extracting circulating anti-A or anti-B antibodies before transplant (Rapaport *et al*. 1968; Eremin *et al*. 2011).

Minor Histocompatibility (mH) antigens

In 1990, Rammensee and his colleagues used high-performance liquid chromatography (HPLC) to separate peptides eluted from MHC molecules, providing the first direct evidence for minor H antigens being peptides (Rotzschke *et al*. 1990; Wallny *et al*. 1990). mH antigens are encoded by polymorphic genes that belong to non-HLA related genes (Dzierzak-Mietla *et al*. 2012). In the context of transplantation, the fact that genetic polymorphisms are present in endogenous proteins expressed by the donor and not the recipient (Bleakley *et al*. 2010) can lead to these proteins being recognised as non-self antigens. Therefore, these proteins can stimulate a donor-specific response (Simpson 1991; Simpson *et al*. 1997; Robertson *et al*. 2007). The male-specific H-Y antigen, encoded by non-HLA related genes, is an example of mHs (Rotzschke *et al*. 1990). This antigen is expressed on the cell surface as several peptides rather than an intact molecule, but either way, it may lead to accelerated graft rejection (Eremin *et al*. 2011).



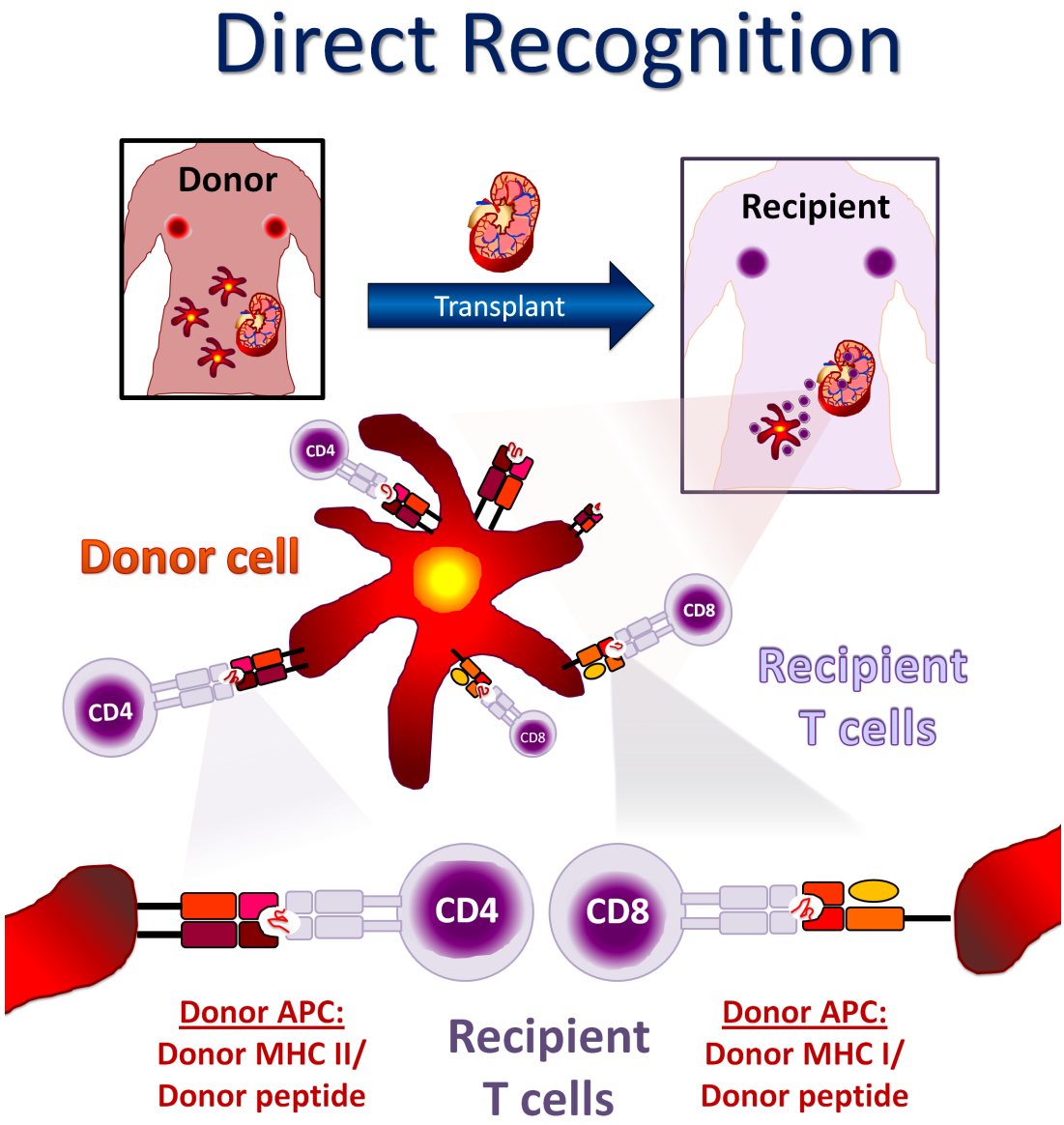
Picture 2: **HLA and ABO system**

1.1.7 Allorecognition in Kidney Transplantation

Allogeneic molecules expressed in the graft can trigger a specific alloimmune response in the recipient when a transplant is performed between genetically disparate individuals of the same species. HLA antigens expressed in donor, but not recipient cells, are the main antigens involved in the immune alloresponse after transplantation (Lechler *et al*. 1982), and can be recognised by the adaptive immune system through three different mechanisms: direct, indirect and semi-direct recognition (Hernandez-Fuentes *et al*. 1999; Herrera *et al*. 2004; Gokmen *et al*. 2008).

Direct pathway: After transplantation, APCs from the donated organ, leave the graft and migrate into regional lymph nodes to activate alloreactive T cells from the recipient (Lindahl and Wilson, 1977; Sandner *et al*., 2003). Donor DCs are the main APCs that primarily trigger the recipient immune response *via* the direct pathway. Thus, depletion of donor DCs leads to loss of immunogenicity that is only restored following addition of donor DCs (Lechler *et al*. 1982). In addition, the alloresponse towards the engrafted tissue is reduced in animals lacking secondary lymphoid tissues (Larsen *et al*., 1990; Lakkis *et al*., 2000).

Once donor DCs induce the activation of alloreactive T cells, these effector T cells migrate back to the graft and destroy the transplanted tissue. This mechanism of allorecognition is known as the direct pathway and involves recipient $CD4^+$ T and $CD8^+$ T cells recognising intact donor MHC II and MHC I molecules on the surface of donor APCs, respectively (Rosenberg *et al*. 1987). This occurs because T cells recognise foreign peptides in a self-MHC restricted manner (Zinkernagel and Doherty, 1974) and as such, the T cell repertoire of the recipient shows a high level of direct anti-donor allo-reactivity (Suchin *et al*., 2001; Baker *et al*., 2001b). This pathway is primarily responsible for eliciting acute allograft rejection (Warrens *et al*. 1994) (Picture 3).



Picture 3: Direct recognition

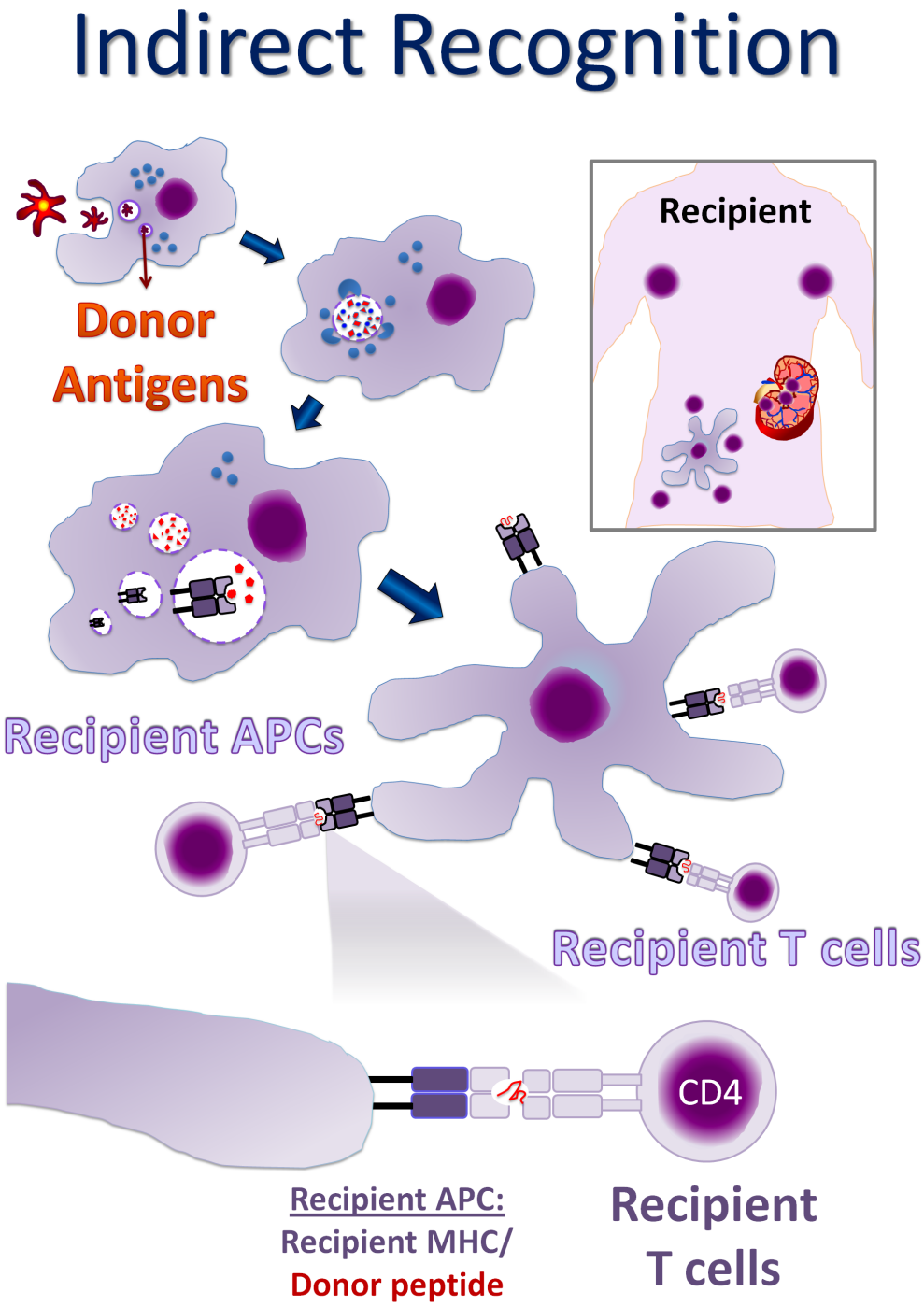
Indirect pathway: The indirect pathway of allorecognition refers to identification of processed peptides of allogeneic histocompatibility antigens presented by self-MHC in a self-restricted manner (Lechler *et al*. 1982; Shoskes *et al*. 1994). Alloantigens shed from a graft are, in general, processed as exogenous antigens and therefore presented by professionals APC, such as B cells, DCs and macrophages, to $CD4^+$ T cells in a self-MHC class II dependent manner (Afzali *et al*. 2007). For example, it has been observed that when direct responses were reduced by CD8 depletion or MHC class I deficiency in transplants models, rapid rejection was observed. Thus, foreign MHC class I peptides were presented *via* self-MHC class II to $CD4^+$ cells (Auchincloss, Jr. *et al*., 1993) Furthermore, when animals elicit indirect rather than direct responses due to immunisation with peptides of allogeneic MHC, a vigorous allograft rejection response was demonstrated (Fangmann *et al*., 1992). All these studies indicate that the indirect pathway has an important role in the allograft rejection.

B cells require the presence of $CD4^+$ T cells in order to induce class switching and to differentiate into antibody-secreting Plasma cells. These T cells recognise peptides derived from antigens internalised by the immunoglobulins on the B cell surface. The presence of class-switched alloantibodies is indicative of T cell help provided *via* the indirect pathway (Lanzavecchia *et al*. 1985; Steele *et al*. 1996). Furthermore, it has been demonstrated that indirect alloantigen presentation by recipients' B cells plays an important role in the efficient progression of acute vascularised allograft rejection. Noorchashm and colleagues have shown that cardiac allograft survival was markedly prolonged in mice with defective B cell antigen presentation when compared to control counterparts (Noorchashm *et al*. 2006).

However, B cells are not the sole APCs required for graft rejection as mice that have B cell or immunoglobulin deficiencies are still able to reject allografted tissues (Nozaki *et al*. 2008). DCs have been shown to be instrumental in allorecognition *via* presentation of peptides from allogeneic donor MHC by in a self-MHC specific manner (Inaba *et al*., 1998). Inaba *et al*. described how DCs process phagocytosed cell fragments onto MHC class II products with unexpected efficacy. They suggest that when migratory donor DCs die upon reaching the lymph node, they are phagocytosed and processed by resident recipient DCs (Inaba *et al*., 1998).

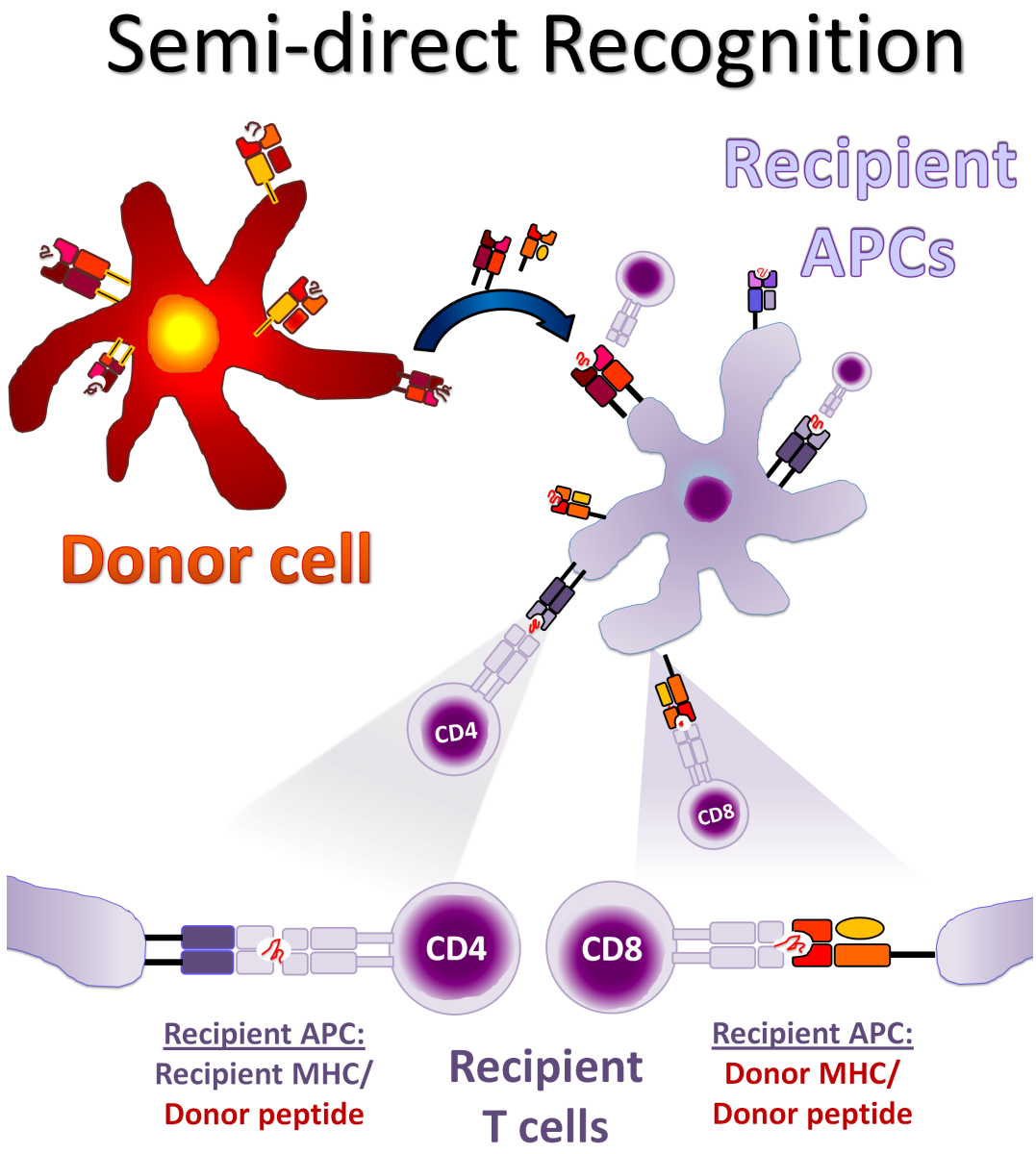
Comparing the efficiency of these two APCs, it has been observed that specific B cells are highly efficient at presenting antigens to T cells due to surface immunoglobulin recognition (Lanzavecchia 1985; Lanzavecchia *et al*. 1985). However, it has also been shown that DCs are as efficient as antigen-specific B cells in presenting tetanus toxoid (TT) to specific T cell clones when activated with granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 (Sallusto *et al*. 1994)., suggesting that both DCs and B cells are equally efficient in antigen presentation to T cells.

The indirect pathway has been linked to graft rejection (Dalchau *et al*., 1992; Liu *et al*., 1992; Liu *et al*., 1996; Tugulea *et al*., 1997), suggesting an important role in long-term alloantigen presentation when donor APC have been exhausted (Vella *et al*., 1997; Hornick *et al*., 2000; Baker *et al*., 2001a), but also in direct allorecognition, as in the absence of direct responses, the indirect pathway alone can also result in rapid acute graft rejection (Auchincloss, Jr. *et al*., 1993) (Picture 4).



Picture 4: Indirect recognition

Semi-direct pathway: Semi-direct allorecognition involves the interaction of recipient T cells with recipient APCs that have acquired intact donor MHC:peptide complexes from donor cells through MHC transfer (Herrera *et al*. 2004). It has been shown that cells from the immune system have the capacity to exchange surface molecules (reviewed in (Smyth *et al*., 2007)). Specifically, DCs are able to acquire intact MHC:peptide complexes from other DCs and endothelial cells, and to present them to alloreactive T cells (Bedford *et al*., 1999; Herrera *et al*., 2004; Smyth *et al*., 2006). Intact MHC can be acquired by direct cell-to-cell contact (Harshyne *et al*., 2001; Herrera *et al*., 2004; Game *et al*., 2005) or *via* the release and uptake of small vesicles ("exosomes") (Denzer *et al*., 2000; Morelli *et al*., 2004). Then, as self-APCs have not only acquired intact donor-MHC, but have also processed these allogeneic histocompatibility proteins and presented them as peptides in the context of MHC class II, these APCs can simultaneously stimulate $CD8^+$ T cells through the direct pathway, and $CD4^+$ T cells through the indirect pathway (Herrera *et al*. 2004) (Picture 5).



Picture 5: Semi-direct recognition

1.1.8 Immunosuppressive Drugs

Controlling the reactions of the immune system is essential to maintain a stable graft function and prevent rejection after transplantation. This control is achieved through the anti-inflammatory effect of immunosuppressive agents (Magee *et al*. 2004). These drugs are extremely heterogeneous and more than one can be used in induction (intense immunosuppressant therapy at the beginning of transplantation), maintenance, and reversal of established rejection (Halloran 2004). Unfortunately, one particular characteristic commonly found in all types of immunosuppressant, is that they all cause adverse side-effects (Niethammer *et al*. 1999).

The main immunosuppressive drugs, with their corresponding side-effects, used in kidney transplantation are as follows:

- **Glucocorticoid:** Steroids penetrate the cell through the membranes to bind to the steroid receptor in the nucleus. They decrease levels of IL-1, TNF-α, GM-CSF, IL-3, IL-4, IL-5 IL-8, nitric oxide, prostaglandins and leukotrienes, inhibit leukocyte emigration from vessels, and activate lymphocyte apoptosis (Niethammer *et al*. 1999). Adverse Effects: Glucose intolerance, hypertension, hyperlipidemia, osteoporosis, osteonecrosis, myopathy, cosmetic defects and growth suppression in children.

- **Cyclosporine:** Cyclosporine binds to cyclophylin (CpN) in the cytoplasm, forming the cyclosporine–CpN complex. This complex binds and blocks the function of the calcineurin (CaN), which in turn, fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc). As a result of this failure, the transport of NF-ATc to the nucleus and the binding of NF-ATc to the nuclear component of the nuclear factor of activated T cells (NF-ATn) are inhibited. The NF-ATc–NF-ATn complex ordinarily binds to the promoter of the *IL-2* gene and initiates IL-2 production. Hence, the presence of cyclosporine prevents IL-2 production by T cells, which is necessary for full T-cell activation (Wiederrecht *et al*. 1993; Ho *et al*. 1996). This drug ultimately inhibits synthesis of IL-2, IL-3, IL-4, GM-CSF, TNF-α in lymphocytes, and proliferation of T and B cells (Niethammer *et al*. 1999). Adverse Effects:

Nephrotoxicity, hyperlipidemia, hypertension, glucose intolerance, hirsutism, gum enlargement.

- **Tacrolimus:** Tacrolimus (FK506) binds to FK506-binding protein (FKBP), forming a FK506–FKBP complex that binds to and blocks calcineurin. The FK506–FKBP–calcineurin complex inhibits the activation of NF-ATc, thus preventing its entrance into the nucleus. This drug ultimately inhibits lymphocyte synthesis of IL-2 (Siekierka *et al*. 1992; Wiederrecht *et al*. 1993). Adverse Effects: Diabetes mellitus, hypertension, hyperlipidemia, and cosmetic defects (less common).

- **Azathioprine:** This pro-drug is converted in the body to the active metabolite 6-mercaptopurine. Azathioprine acts to inhibit purine synthesis necessary for the proliferation of cells, particularly leukocytes and lymphocytes. It also reduces immunoglobulin production, monocytes activation and cytokine production (Niethammer *et al*. 1999; Maltzman *et al*. 2003). Adverse Effects: Predominantly bone marrow suppression, hepatitis and/or pancreatitis (rare).

- **Mycophenolate Mofetil:** This pro-drug is immediately converted to mycophenolic acid (MPA), an inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is the first of two enzymes responsible for the conversion of inosine monophosphate (IMP) to guanosine monophosphate (GMP), which is normally converted to GDP, GTP, and dGTP. Measurements show that MPA causes a reduction of GTP and dGTP in lymphocytes, but not neutrophils (Ransom 1995; Niethammer *et al*. 1999). Mycophenolate Mofetil inhibits leukocyte proliferation and GTP-dependent metabolic events, and is relatively more selective for lymphocytes than azathioprine. Adverse Effects: Bone marrow suppression, nausea, abdominal pain, diarrhea. Invasive CMV disease is more common than with azathioprine.

- **Sirolimus:** This drug is an antifungal agent that forms an immunosuppressive complex with intracellular protein, FKBP12. This complex blocks the activation of the cell-cycle-specific kinase, TOR. The downstream events that follow the inactivation of TOR result in the blockage of cell-cycle progression at the juncture of G1 and S phase, therefore, sirolimus inhibits leukocyte proliferation

(Sehgal 2003). Adverse Effects: Bone marrow suppression, hyperlipidemia, diarrhea and interstitial pneumonitis (rare).

- **Chimeric monoclonal antibodies** Basiliximab and Daclizumab are monoclonal antibodies against the IL-2 receptor (Pascual *et al*. 2001); Campath-1H, or Alemtuzumab, is a humanised IgG1 monoclonal antibody against CD52, a protein expressed on a variety of lymphoid neoplasm and most human mononuclear cell subsets (Alinari *et al*. 2007); Anti-thymocyte globulin (ATG) is a polyclonal antibody capable of binding to various immune-cell subsets such as T cells, B cells, natural killer cells, monocytes/macrophages, neutrophils and DC (Hoegh-Petersen *et al*. 2013); OKT3 is a murine monoclonal antibody against CD3, part of a multimolecular complex found only on mature T cells and medullary thymocytes, and block T-cell receptor function (Norman 1995). Adverse Effects: Cytokine release syndrome, and post-transplant lymphoproliferative disorder (PTLD) for ATG.

1.1.9 Clinical events secondary to immune alloresponses

Even though immunosuppressive drugs improve graft survival, several factors such as HLA antigen matching, production of donor-specific antibodies, co-morbid conditions, age, organ origin and recipient quality of life are also important in understanding the different outcomes after transplantation:

Hyper acute rejection: Also known as pre-formed antibody mediated rejection. This occurs immediately after revascularisation of the graft and is characterised by widespread glomerular capillary thrombosis, and necrosis. Both effects are caused by the interaction of lymphocytotoxic recipient antibodies with donor HLA and other endothelium associated donor-antigens (Solez *et al*. 1993). Once these antibodies recognise their targets, the complement system is triggered in the endothelium, activating coagulation cascade *via* complement. Both sequential activations induce thrombus formation in the capillaries of the kidneys (Collins *et al*. 1999). Antibody-mediated rejection is frequently associated with graft loss because of its irreversible nature, and the only option of treatment is graft removal (Chinen *et al*. 2010).

Accelerated rejection: Also known as delayed hyper acute rejection. This occurs a few days after transplantation and is characterised by haemorrhage-induced

vascular disruption mainly caused by non-complement-fixing antibodies (Solez *et al*. 1993). The treatment of choice to reverse this type of rejection is anti-lymphocyte reagents (Chinen *et al*. 2010).

Acute rejection: This is most frequently observed in the first three months after transplantation, and is characterised by tubulitis and vasculitis (Chinen *et al*. 2010). Tubulitis is defined as the invasion of tubules by mononuclear cells (lymphocytes or macrophages) across the tubular basement membrane (Ganji *et al*. 2007; McKay *et al*. 2010); and vasculitis occurs when antibodies induce inflammation of the blood vessels. Other causes of acute allograft dysfunction include interstitial nephritis, infection, acute tubular necrosis, toxicity by drugs, and obstruction in the urinary tract (Ganji *et al*. 2007). Diagnosis can only be made by allograft biopsy. High-dose pulses of steroids and lymphocyte depleting agents reverse the T cell response in about 80% to 90% of patients.

Chronic rejection: This occurs from months to years following transplantation. It is characterised by graft arterial occlusions induced by collagen production by fibroblasts, and smooth muscle cell proliferation. This fibrosis can cause ischemia and cell death in the graft (Solez *et al*. 1993). Chronic rejection has been associated with increased immune responses, mediated by indirectly activated T cells (Baker *et al*. 2001; Baker *et al*. 2001). This process is usually treatment resistant, although progression may be delayed by immunosuppressive drugs (Chinen *et al*. 2010).

Stable function: This is a "host–graft adaptation" state characterised by reduced donor-specific responses and low risk of rejection, in the presence of immunosuppressive therapy, after a successful transplant (Starzl *et al*. 1963).

Tolerance: The most widely accepted definition for transplantation tolerance is the absence of a destructive immune response to the allograft without the need for generalised immunosuppression (Womer 2005).

1.2 Immunological Tolerance in Kidney Transplantation

1.2.1 Transplantation Tolerance

Despite the immune down-regulation obtained through the use of immunosuppressive drugs in graft maintenance, side-effects still remain an important concern in kidney transplantation. An ideal outcome for patients would be to develop spontaneous tolerance to the foreign organ, or tolerance induced by new therapeutic strategies.

Tolerance has been generally defined as a state of specific immunologic unresponsiveness to the antigens of the graft, in the absence of immunosuppression (Nickerson *et al*. 1994; Dong *et al*. 1999). More specifically, in animal models, tolerance is the absence of acute rejection with indefinite graft survival and acceptance of a second test graft from the original donor, while maintaining the ability to reject a third party graft (Dong *et al*. 1999). Although the former ideas are correct, the definition of tolerance in the clinic should also include: absence of donor-specific alloantibodies, no signs of destructive lymphocyte infiltration in allograft biopsies and, if systemic tolerance is sought, proof of donor-specific unresponsiveness with recovered third party responses in functional assays *in vitro* (Thomas *et al*. 2001).

1.2.2 Basis of Tolerance

The induction of self-tolerance is a multistep process that begins in the thymus during foetal ontogeny (Geenen *et al*. 2001) and continues in the thymus for T cells, and in the bone marrow for B cells, during central-tolerance development. This process is responsible for the unresponsiveness of T and B cells to self-molecules, and involves a negative and positive selection. Negative selection is when "self-reactive" T and B cells are commanded to undergo apoptosis because they become activated after contact with self-antigens, whilst positive selection is when T cells are able to recognise peptides only in the context of self MHC I and II (Murphy *et al*. 2012). Peripheral tolerance occurs after the T and B cell maturation process. T cells and B cell then leave the thymus and the bone marrow, respectively, and enter into peripheral circulation. Several mechanisms control and maintain central and peripheral self-tolerance.

1.2.3 Mechanism of Tolerance in Transplantation

Allograft tolerance mechanisms are the same as those applied to self-tolerance: deletion, ignorance (Fehr *et al*. 2004), anergy (Dong *et al*. 1999) and regulation/suppression. This has been amply studied in animal models of transplantation.

Deletion of self-reactive T and B cells is performed during the maturation process in central tolerance (Lechler *et al*. 2003). The participation of deletion in peripheral tolerance is not well established but studies on Veto cells, a subset of suppressor cells, demonstrate that T cells were led to inactivation or deletion after Veto cell recognition (Thomas *et al*. 1991; Thomas *et al*. 1994).

Ignorance can occur in two principal situations: when donor-antigens cannot reach the recipient's lymph nodes, or when the immune cells from the recipient cannot invade the foreign graft. In both cases, the immune system from the recipient remains ignorant to donor-antigens. This applies to non-vascularised grafts and graft without lymphatic drainage (Fehr *et al*. 2004).

Anergy is a state of functional inactivation in which antigen-specific T cells are present as functionally inert, or unable to respond cells (reviewed in (Lechler *et al*. 2001)). This impairment can be observed as failures in T cell proliferation, activation and cytokine production (Schwartz 1990; Dong *et al*. 1999; Womer 2005). Absence of co-stimulatory signals as CD2:LFA-3, LFA-1:ICAM-1 and CD28:CD86/CD80, can also induce cell unresponsiveness. Another important protein in the induction of anergy is CTLA-4. Unlike CD28-CD80/86 interaction, CTLA-4-CD80/86 interaction down-regulates the activation of the immune response. CTLA-4 also has higher affinity for the CD80/86 co-stimulatory molecules than CD28, therefore its presence is able to interrupt the interaction between CD80/86 and CD28 (Dong *et al*. 1999).

Regulation/suppression: Regarding peripheral tolerance, the role of regulatory or suppressor cells seem to be crucial in graft acceptance. Different cells have been shown to be involved in peripheral tolerance:

- **$CD4^+$ T regulatory cells**

Regulatory T cells (Tregs) maintain actively immunological self-tolerance for life. In the context of transplantation, multiple populations with different phenotypes and

regulatory mechanisms have been described as key players in graft acceptance and stable function maintenance. One of the populations described is the natural occurring subset of $CD4^+$ T cells that arise during T cell development in the thymus (Walsh *et al*. 2004)**.** These endogenous Tregs leave the thymus as a functionally unique and mature T cell subpopulation. Most endogenous $CD4^+$ Tregs constitutively express high levels of CD25, the alpha-chain of the IL-2 receptor (Sakaguchi *et al*. 1995); and the transcription factor, Foxp3 (Hori *et al*. 2003). The mechanisms used by these cells to suppress effector T cell proliferation *in vitro* involves: cell contact–dependent mechanisms (Takahashi *et al*. 1998), CTLA-4 mediated signalling (Takahashi *et al*. 2000), GITR (Shimizu *et al*. 2002), IL-10 (Hara *et al*. 2001) and TGF-β production (Josien *et al*. 1998).

In vivo, experiments in organ transplantation demonstrated that pro-inflammatory $CD45RB^{high}$ T cells were capable of inducing rejection in grafted T cell-depleted mice. However, when natural Tregs were co-transferred with $CD45RB^{high}$ T cells into these mice, Tregs prevented graft rejection by inhibiting the onset of the immune system activation (Powrie *et al*. 1994; Hara *et al*. 2001).

A second population of regulatory $CD4^+$ cells could be defined as inducible $CD4^+$ Tregs. Unlike natural Tregs, inducible Tregs have acquired their immunosuppressive capacity in periphery. Th3 and Tr1 cells are recognised as inducible Tregs; both types seem to function independently of cell-cell contact and the main regulatory mechanism performed is cytokine secretion. The first population is characterised by their capacity to secrete TGF-β (Chen *et al*. 2003), and the second population, described and studied by Roncarolo's groups, exhibited the ability to produce IL-10 (Roncarolo *et al*. 2006). The relevance of these cytokines can be explained by the fact that TGF-β has been found *in situ* in tolerated grafts. This means that TGF-β was involved in the suppression of the allograft rejection, demonstrating that TGF-β-producing Tregs were participating in the maintenance of tolerance (Josien *et al*. 1998). IL-10 is a regulatory cytokine with an anti-proliferative role. It has been shown that IL-10 inhibits T cell proliferation and IL-2 production in humans (Taga *et al*. 1992). Blockade of IL-10 by the administration of either IL-10-specific, or IL-10-receptor-specific antibodies abrogated the suppression of skin-allograft rejection by Tregs cells that were present during both the induction (Kingsley *et al*. 2002), and maintenance of tolerance (Hara *et al*. 2001). Induced

regulatory T cells have been proposed as a major player in maintenance of tolerance after transplantation (Walsh *et al*. 2004).

- **$CD8^+$ T cells**

$CD8^+$ T cells are not conventional regulatory cells, but specific subsets have been reported to be involved in the maintenance of tolerance. It was demonstrated that $CD8^+CD28^-$ T cells suppressed the alloresponse of $CD4^+$ T cells in both primates and rodents (Colovai *et al*. 2001; Liu *et al*. 2001). It was also demonstrated that IL-10–producing $CD8^+$ Tr cells, induced by allogeneic CD40L–activated plasmacytoid DCs, displayed a poor secondary-proliferative and cytolytic response (Gilliet *et al*. 2002). Other groups described that novel human $CD8^+$/TCR$\alpha\beta^+$ T cells, with a specific regulatory phenotype and non-cytolytic function, have the ability to inhibit the immune response *in vitro* by secreting IL-4, IL-5 and IL-13 (Jarvis *et al*. 2005). Li and colleagues, using a model of transplantation tolerance mediated by $CD8^+$ Tregs following CD40Ig treatment in rats, showed that the accumulation of tolerogenic $CD8^+$ Tregs and plasmacytoid DCs in allograft and spleen was associated with tolerance induction in vascularised allograft recipients (Li *et al*. 2010). Finally, a recent study reported that polyclonal naïve $CD8^+$ T cells stimulated with allogeneic DCs in the presence of IL-2, TGF-β1 and retinoic acid, have the ability to proliferate robustly and differentiate into allosuppressive $CD8^+Foxp3^+$ T cells. These cells were capable of enhanced expression of CD103, CTLA-4, and CCR4, and suppressing T cell proliferation and IFN-γ production in an alloantigen-specific and contact-dependent manner. They also induced $CD4^+CD25^+Foxp3^+$ Tregs in a TGF-β-dependent fashion, and both, $CD8^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ Tregs, protected full MHC-mismatched skin allograft (Lerret *et al*. 2012).

- **Invariant Natural Killer T (NKT) cells**

NKT cells are characterised by co-expression of NK receptors, and a single invariant TCR (Seino *et al*. 2001). They represent <1% of total T cells in blood and ~30% of all T cells in the marrow. Experimental results demonstrated that transplantation of sorted $CD4^+$ and $CD8^+$ T cells from the blood or marrow were able to induce acute lethal graft *versus* host disease (GVHD). Interestingly, when $NK1.1^+$ T cells were present in this model, the disease was suppressed. The marrow $NK1.1^+$ T cells secreted

high levels of both IFN-γ and IL-4, whereas $NK1.1^-$ T cells only secreted high levels of IFN-γ. The suppressive activity of the $NK1.1^+$ T cells was dependent on their secretion of IL-4, as $NK1.1^+$ T cells from IL-$4^{-/-}$ donor mice failed to ameliorate, and even worsened, GVHD, compared to wild-type mice. Together, these results demonstrate that GVHD is regulated by the differential cytokines produced by $NK1.1^-$ and $NK1.1^+$ T cells (Zeng *et al*. 1999). In humans, $V\alpha24^+V\beta11^+$ $CD4^+$ NKT cells isolated from peripheral blood secrete substantial amounts of IL-2 after stimulation with DC and α-Galactosylceramide. Since IL-2-producing NKT cells induced the expansion of anergic $CD4^+CD25^+$ cells, this indicates that NKT cells promote $CD4^+CD25^+$ regulatory cell proliferation (Jiang *et al*. 2005).

One study looked at the role of NKT cells in allograft rejection and tolerance, within a murine model of transplantation. They found that NKT cells played a critical role in the induction of vascularised cardiac allograft tolerance by blockade of lymphocyte LFA-1, ICAM-1 or CD28/B7 interactions (Seino *et al*. 2001). A second study focussing on transplantation, observed that host NKT cell were required for heart graft acceptance. Host secretion of IL-4 and IL-10 were critical in determining the induction of tolerance to both bone marrow and heart grafts in this model (Higuchi *et al*. 2002). The interactions between NKT cells and Tregs also facilitated tolerance in a model of combined organ and bone marrow transplantation. Tolerance was dependent on IL-10 production by Tregs, which in turn, was dependent on IL-4 production by NKT cells (Hongo *et al*. 2012).

- **Dendritic cells (DCs)**

DCs are professional APCs that modulate the outcome of the immune response towards immune activation or tolerance. The maturation status and the stimulation used by DCs present in the grafted tissue are undoubtedly critical factors in the outcome of the alloimmune response. Immature DCs residing in peripheral tissues, such as epidermal Langerhans cells, failed to induce T cell activation. This was due to moderate levels of expression of MHC class II, and very low levels of co-stimulatory molecules (Lutz *et al*. 2000). Immature DCs can also induce T cell unresponsiveness through the specific DCs markers 33D1 and DEC-205, and through a sub-optimal stimulation of effector T cells, due to a low expression of T cell co-stimulatory factors and pro-inflammatory cytokines (Hawiger *et al*. 2001).

DCs can act as tolerogenic cells because of the expression of the inducible enzyme indoleamine 2,3-dioxygenase (IDO); this enzyme is responsible for the degradation of tryptophan, an amino acid essential in cell proliferation. DCs can also act as tolerogenic cells by expression of the inducible co-stimulator ligand (ICOS-L), and/or the programmed death 1 ligand (PDL-1); both mediate Treg development, and suppression of self- and alloreactive cells (Gehrie *et al*. 2011).

- **B cells**

The role of B cells in kidney transplantation has been focused mainly in B cell-derived-Plasma cells and the production of donor-specific antibodies (DSA) (Terasaki *et al*. 2005). Current theories explaining the role of B cells in tolerance or rejection, now consider the cytokine production and the antigen-presenting role of B cells as important mechanisms. B cells in mice transplant models have been shown to actively participate in allograft rejection when T-cells were depleted or suppressed, but the depletion of B cells could not prevent a T-cell driven rejection (Brandle *et al*. 1998). In another study, passive transfer of donor-specific immune serum to B cell-deficient recipients significantly accelerated allograft rejection (Zarkhin *et al*. 2008). In human translational research, certain studies strongly associate B cells with severe graft rejection and steroid resistance (Sarwal *et al*. 2003; Hippen *et al*. 2005; Tsai *et al*. 2006). However, other studies show that $CD20^+$ B cells are not responsible for poor outcomes or glucocorticoid resistance (Doria *et al*. 2006; Bagnasco *et al*. 2007; Kayler *et al*. 2007; Mengel *et al*. 2007). The same applies to anti-CD20 therapies (Becker *et al*. 2004; Alausa *et al*. 2005; Genberg *et al*. 2006; Lehnhardt *et al*. 2006; Faguer *et al*. 2007). As the role of B cells in transplantation tolerance is an important topic in this thesis, it will be discussed in more detail in the following chapter and in the discussion.

1.2.4 Immunological Tolerance in Kidney Transplantation

Developing transplantation tolerance, long-term graft acceptance without immunosuppressant therapy, is the ideal outcome for kidney transplant recipients. Even when immunosuppressive drugs control acute rejection and improve graft survival in organ transplantation, their side-effects (increased risk of infection, cardiovascular diseases, cancer, and renal failure) (Soulillou *et al*. 2001; Fishman 2007) maintain recipients in an unpleasant and undesirable clinical state.

In 1953, Billingham *et al*. demonstrated the feasibility of "actively acquired tolerance" in a neonatal mouse model. They demonstrated that injection of foreign antigens in mice at foetal stage induced skin graft tolerance to the same donor at adult stage. They proposed the definition of true tolerance to be *stable graft function without histological signs of rejection in an immunocompetent host, in the absence of immunosuppression, accepting a second graft of the same donor, but rejecting one from a third-party graft* (Billingham *et al*. 1953).

Decades later, the concepts of intrathymic injection of alloantigens (Ildstad *et al*. 1985; Posselt *et al*. 1990) and mixed chimerism (Kurtz *et al*. 2004) were introduced in the induction of central tolerance to foreign antigens.

Intrathymic injection of alloantigens was used to achieve transplantation tolerance by Remuzzi *et al*. They observed no donor-specific response after the injection of isolated glomeruli from Brown-Norway rat kidneys into Lewis rats (Remuzzi *et al*. 1991). The same group followed on from this with a human study, by injecting donor cells into the thymus before heart transplantation; however, this technique was insufficient to induce the long-term graft survival observed in mice (Remuzzi *et al*. 1995).

Mixed chimerism was defined by David Sachs and Megan Sykes as the state in which donor and host haematopoietic elements of multiple lineages coexist at levels detectable by flow cytometry (Sykes *et al*. 1999; Sykes *et al*. 2001). This technique consists in the engraftment of allogeneic bone marrow in the recipient compartment, to provide the recipient with a permanent source of donor-antigens. Allogeneic T cell maturation is a key mechanism in mixed chimerism as engrafted donor T cells emerging from recipient bone marrow are not recognised as foreign cells by the central tolerance

system during negative/positive selection. In turn, the recipient identifies them as self-T cells. Reactive cells against both self-peptides and allogeneic peptides from the engraftment are then eliminated before entering into circulation as mature T cells. This intrathymic deletion of donor-reactive thymocytes was shown to be the dominant mechanism for the maintenance of tolerance in mixed chimerism transplantation (Kurtz *et al*. 2004).

Although specific tolerance to the donor was achieved due to a constant source of donor-antigen, no evidence of peripheral mechanisms were found in these models (Khan *et al*. 1996). Mouse models demonstrated improvements in graft survival with mixed chimerism, without a complete lethal ablation of host's immune system (Cobbold *et al*. 1986) or using a non-lethal conditioning regimen in skin allograft model (Sharabi *et al*. 1989).

In humans the phenomenon of mixed chimerism was observed in two HLA-matched bone marrow transplanted patients who, a few years later, were able to accept a kidney graft from the same donor without immunosuppressant therapy (Sayegh *et al*. 1991).

Scandling *et al*. developed a clinically relevant, non-myeloablative regimen to induce tolerance in human kidney transplantation. The first clinical case reported was a kidney post-transplanted recipient maintained with a regimen of total lymphoid irradiation plus injection of rabbit anti-thymocyte immunoglobulins. Stable mixed chimerism and stable graft function, without immunosuppressant regimens, were observed in this patient. The immune responses did not suffer any counterproductive effect. This study demonstrated that persistent mixed chimerism and graft tolerance are possible without the development of GVHD (Scandling *et al*. 2008).

Kawai *et al*. reported mixed chimerism in patients with stable renal allograft-function, after complete withdrawal of immunosuppressive therapy (Kawai *et al*. 2008). Unlike Scandling's study, they used bone marrow and kidney transplantation from HLA mismatched donors. All patients displayed stable creatinine levels and stable graft function, despite the presence of anti-donor HLA II antibodies and C4d deposits in three of the four recipients. High-level of Foxp3 expression was observed in tolerated grafts, suggesting regulatory T cells were involved in the maintenance of tolerance. Authors were unable to detect mixed chimerism after 14 days.

Not only has central tolerance been targeted to induce graft acceptance, peripheral tolerance has also been shown to be an interesting mechanism to regulate the immune response in order to prevent graft rejection. Inhibitors of T cell signalling such as cyclosporin A, tacrolimus and sirolimus have been used to improve transplantation outcomes and graft survival, despite their undesirable adverse effects (Galon *et al*. 2002). Monoclonal antibodies have been also used to achieve peripheral tolerance in animal models and humans (Table 1).

Monoclonal antibody	**Bind to**	**Function**	**References**
Anti-thymocyte globulin	Thymocyte globulin	Deplete alloreactive peripheral T cells	(Strober *et al*. 1984; Strober *et al*. 1989)
OKT3	Anti-CD3 monoclonal antibody	Block activation of T cell in vitro	(Cosimi *et al*. 1981)
anti-IL-2R	IL-2 receptor	Inhibit proliferation of activated T cells in vitro	(Kirkman *et al*. 1985; Shapiro *et al*. 1987)
(33B1.3)	Rat monoclonal antibody that blocks the α and β chain association of the IL-2 receptor.	No interaction between IL-2 and its receptor. Prevent acute rejection.	(Soulillou *et al*. 1990) (Brennan *et al*. 2006) (Bluestone *et al*. 2008)
Alentuzumab (Campath-1H)	Humanised rat monoclonal antibody. It binds CD52, a glycoprotein expressed by T and B lymphocytes, monocytes and granulocytes.	Massive depletion of peripheral lymphocytes. Treatment of lymphoma. Induction of kidney transplantation tolerance. Decrease acute rejection and opportunistic infections. Increase the number of Tregs	(Hale *et al*. 2002) (Calne *et al*. 1998) (Ciancio *et al*. 2008)
Belatacept (LEA29Y)	Selective costimulation blocker. Binds surface costimulatory ligands (CD80 and CD86) of APCs.	Blockade of second signal inducing death and anergy of effector T cells.	(Schwartz 1990) (Sayegh *et al*. 1998) (Vincenti *et al*. 2005) (Bluestone *et al*. 2008)
Anti-CD28	Selective CD28 blocker	Reduce alloreactivity and increased the pool of peripheral T regulatory cells	(Poirier *et al*. 2012)

Table 1: Monoclonal antibodies

1.2.5 Spontaneous Tolerance in Human Kidney Transplantation

Development of spontaneous tolerance in humans, characterised by a stable graft function after quitting immunosuppressive therapy, has been reported in the last decades, mainly in liver transplantation (Lerut *et al*. 2006; Castellaneta *et al*. 2010; Mazariegos 2011; Sanchez-Fueyo *et al*. 2011), but also in kidney transplantation (Roussey-Kesler *et al*. 2006). Identification of this spontaneous tolerance was a completely random event in humans, as doctors observed that some of the patients who deliberately decided to stop immunosuppression, displayed a stable function a long time after immunosuppressants were withdrawn.

In 2010, three major studies were published that focused on identifying biomarkers of tolerance in kidney transplant patients, Pallier *et al*. (Pallier *et al*. 2010) from France; Indices of Tolerance (IOT), (Sagoo *et al*. 2010) from Europe; and Immune Tolerance Network (ITN), (Newell *et al*. 2010) from the USA. All studies were carried out independently of each other, and all showed that tolerant patients displayed an expansion of peripheral blood B cells, an absence of donor-specific antibodies and an increased expression of multiple B cell-related genes. No strong evidence of other subsets was observed in tolerant recipients among these three studies.

1.2.6 B cells as a signature of Spontaneous Tolerance in Kidney Transplantation

In 2007, Brouard *et al*. performed the first study trying to identify biomarkers of tolerance from peripheral blood samples. The idea was to use these biomarkers to determine tolerance in immunosuppressed patients with stable graft function (Brouard *et al*. 2007). They measured gene expression using microarray analysis and identified a "tolerant fingerprint" of 49 genes. The group found that in tolerant recipients, there was an over expression of genes encoding proteins presented in the B cells, such as CHEK1, PIM2, LCK, ZAP70, IKBKB, PDGFRA, and LPXN (Brouard *et al*. 2007).

The same group continued with the biomarkers of tolerance investigation but this time, focused solely on the B cell population. They compared peripheral B cell subsets from patients with stable graft function, chronic rejection, healthy volunteers and tolerance, and found that tolerant recipients exhibited a significant increase in absolute B cell numbers and frequency of total B cells, particularly activated Memory

and early Memory B cells. They suggested that B cells exhibited an inhibitory phenotype. Evidence for this was based on the decreased FcγRIIA/FcγRIIB ratio and high expression of CD1d and CD5 observed in these cells. They also found a significantly enriched transcriptional B-cell profile in cells from peripheral blood (Pallier *et al*. 2010).

In the same year two multicentre studies, the European IOT study (Sagoo *et al*. 2010) and the USA ITN study (Newell *et al*. 2010), reported a clear B cell signature in kidney transplant tolerant patients. The IOT and INT studies included 11 and 19 operationally tolerant recipients, respectively. Both studies found an expansion of peripheral blood B cells and an over-expression of multiple B cell differentiation genes. They also used the following set of genes to distinguish tolerant from non-tolerant recipients: *MS4A1, FCRL1, FCLR2, CD79B, TCL1A, HS3ST1, SLC8A1, TLR-5, SH2D1B* and *PNOC* (IOT study); *IGKV1D-13*, *IGKV4-1* and *IGLL1* (ITN study). Finally, the ITN study also found an up-regulation of *CD20* mRNA in urine sediment cells, and elevated numbers of peripheral blood Naïve and Transitional B cells in tolerant participants, compared with those receiving immunosuppression.

The former findings in spontaneous tolerance described in the last paragraphs, were the preliminary data used to structure the main idea of this thesis. Identification of spontaneous tolerance would also be a beneficial clinical diagnosis for some kidney transplant patients with stable function. For all these reasons, the role of the B cells and their subsets in tolerance was extensively studied in this thesis. However, the fact that B cells have always been associated with antibody production, and therefore, to chronic rejection cannot be ignored. Naturally, this poses the question: why could B cells be playing a regulatory role in tolerance? In order to understand and answer this question, the following subchapter will introduce B cells and the different subsets found in peripheral blood, explain the different roles of the B cell in the immune response, and most importantly, will describe immune regulation displayed by these cells in transplantation tolerance.

1.3 B cells as part of the Immune System

The immune system is a complex structure of organs and cells that recognise foreign pathogens in the body, generating a defensive response to protect the organism. The interaction between the immune organs, immune cells, signalling pathways and proteins are essential to maintain this system in operation.

The defensive response of the immune system can be academically divided into the innate and adaptive immune response. The innate immune response is a rapid response that initiates the first recognition of pathogens without specificity, and the adaptive immune response is a specific response of late-onset that requires pathogenic antigen processing. The main cells involved in these two responses come from precursors from the bone marrow, where they mature and differentiate into several cell lineages to finally form the different types of leukocytes. The myeloid lineage conceives the innate participants, such as macrophages, granulocytes, mast cells and DCs (Murphy *et al*. 2012), whereas the lymphoid lineage conceives lymphocytes and NKs. Lymphocytes are the main players in the adaptive response, while NKs participate mainly in the innate response (Murphy *et al*. 2012).

1.3.1 B cells

B cells are the key players of the humoral response in the immune system. Their main role is antibody production by B cell derived-Plasma cells, but besides this function, B cells can play a role as professional APCs. This is because they process and present antigens in the context of MHC class II, and can regulate the immune response through cytokines secretion.

1.3.2 The origin of the B cell

B cells originate from the bone marrow when a multi-potent hematopoietic stem cell is differentiated into a common-lymphoid progenitor (CLPs) after successive rounds of lineage fate restriction, commanded by transcription factor Ikaros and Purine box factor 1.

CLPs then undergo the first round of differentiation, into the B cell lineage through transcription factors E box binding protein 2A (E2A) and early B cell factor-1

(EBF1). These two transcription factors induce transcription factor Pax-5, that in turn, promotes genes for the B cell co-receptor Igα, CD19 and B-cell linker protein (BLNK). These allow the CLPs to differentiate into Pro-B cells (Ramirez *et al*. 2010). Rearrangement of D-J_H gene segment starts in the early Pro-B cell, while V_H-DJ_H rearrangement occurs in the late Pro-B cell.

After VDJ_H rearrangement, the cell begins to expresses a pre-B cell receptor (BCR) and a complete immunoglobulin μ heavy chain, before developing into a Pre-B cell (Allman *et al*. 1999). E2A and EBF1 then induce the expression of λ5 and VpreB, and together, these two proteins form the surrogate light chain; λ5 replaces the C domain, whereas VpreB replaces the V domain, plus an extra region. The pre-BCR is completed when the heavy chain, the surrogate light chain and the heterodimer Igα/Igβ are assembled in the surface of the cell (Monroe 2006).

The expression of this pre-BCR significantly reduces λ5 production and induces the rearrangement of the light-chain locus. The light-chain lacks D segments; therefore rearrangement occurs only in the V-J_L gene segment. VJ_L-C_L assembling completes the light-chain of the BCR, and both the light and heavy chains form the proper BCR. This is located on the surface of the B cell, and the cell becomes an immature B cell during its permanence in the bone marrow.

1.3.3 Mice B cells phenotype

B cells emerge from the bone marrow as an immature B cell. The first immature population that arrives in periphery is called Transitional 1 (T-1), or newly formed (NF) B cell, and it is characterised by the expression of $B220^+$ $CD19^+$ $sIgM^{high}$ $sIgD^{-/low}$ $CD23^-$ $AA4^+$ and $CD21/35^{-/low}$. T1 cells can become Transitional 2 (T-2) B cells, characterised as $B220^+$ $CD19^+$ $sIgM^{high}$ $sIgD^{high}$ $CD23^+$ $AA4^+$ and $CD21/35^{low}$, and T-2 can become Transitional 3 (T-3) B cell characterised as $B220^+$ $CD19^+$ $sIgM^{low}$ $sIgD^{high}$ $CD23^+$ $AA4^+$ and $CD21/35^{low}$ (Allman *et al*. 2008). These three populations of transitional cells represent the first maturation process of the B cells that enter into the periphery for the first time. Even when the daily output of immature B cells is roughly 5-10% of the total B cells, the majority of these do not even reach the follicles; they normally die and do not continue with the maturation process. A small fraction of immature B cell escapes the deadly fate and become part of the Naïve population of B

cells that can be divided into follicular (FO) B cells, marginal zone (MZ) B cells and long-lived B-1 B cells (Casola 2007).

FO B cells can be classified as follicular type I ($B220^{+}$, $CD19^{+}$, $sIgM^{low}$, $sIgD^{high}$, $CD23^{+}$, $CD21/35^{int}$) derived from T2 and T3 B cells, and follicular type II ($B220^{+}$, $CD19^{+}$, $sIgM^{high}$, $sIgD^{high}$, $CD23^{+}$, $AA4^{-}$, $CD21/35^{int}$) derived from T2 B cells. FO B cells reside in follicles and participate in both T-cell dependent and T-cell independent immune responses.

MZ B cells ($B220^{+}$, $CD19^{+}$, $sIgM^{high}$, $sIgD^{low}$, $CD23^{-}$, $AA4^{-}$, $CD21/35^{high}$ $CD1d^{+}$) are derived from the MZ precursor (PMZ) ($B220^{+}$, $CD19^{+}$, $sIgM^{high}$, $sIgD^{low}$, $CD23^{+}$, $AA4^{-/low}$ $CD21/35^{high}$ $CD1d^{+}$), which in turn, is derived from T2 B cells. MZ B cells reside in the marginal zone between the red and white pulp, and because they are located close to the marginal sinus, they respond rapidly to blood-borne pathogens (Pillai *et al*. 2005).

The decision that determines the final B cell lineage from T2 cells is given by the BCR signalling. *If the BCR reacts fairly well with a self-antigen, this B cell is induced to differentiate in a Btk-pathway-dependent manner into an FO B cell. If BCR reacts poorly or not at all with a cognate self-antigen, it continues to survive because of BAFF and constitutive BCR signalling, but in the absence of stronger BCR stimulation it is receptive to inductive signals that drive it to an MZ B cell fate* (Pillai *et al*. 2005).

B-1 cells come from a progenitor population detected in foetal liver and foetal bone marrow, defined as $Lin^{-}CD45R^{lo\text{-}neg}CD19^{+}$, that generates functional B-1a ($sIgM^{hi}CD11b^{+}CD5^{+}$) or B-1b ($sIgM^{hi}CD11b^{+}CD5^{-}$) B cells, but not the rest of the B cell subsets (Montecino-Rodriguez *et al*. 2006). B-1 cells reside in the peritoneal and pleural cavities, and contribute to the generation of IgM responses to T-independent antigens (Allman *et al*. 2008).

1.3.4 Human B cells phenotype

Similarly to mouse development, human immature B cells leave the bone marrow and enter into the circulation as B cells with a transitional phenotype. In bone marrow and cord blood, the immature B cell phenotype was characterised by Sims and

colleagues, who found that markers such as IgM, IgD, CD20, CD24 and CD38 were highly expressed; markers such as CD5, CD10 and CD1d were equally expressed; and markers such as CD21, CD23 and CD27 were poorly expressed or not expressed at all in immature B cells (Sims *et al*. 2005). Once the immature phenotype was characterised, the same group evaluated the same markers in B cells from peripheral blood, and a small population with the same phenotype as immature B cells was found in circulation. Because of the high levels of IgM but low levels of CD21, CD23 and CD62L expressed by these cells, they were associated with the transitional phenotype exhibited in mice. Probably for this reason, this human population was also defined as Transitional B cells. They were distinctively distinguished from mature Naïve B cells by their high expression of CD10, CD24 and CD38, and the low expression of CD44.

Naïve B cells gradually lose the high expression of CD38 and CD24, and are therefore recognised as $IgM^{+}IgD^{+}CD19^{int}CD20^{+}CD38^{int}CD24^{+}CD27^{-}$ cells. Following this, Memory B cells up-regulate CD27, and they are recognised as IgD^{-} $CD19^{int}CD20^{+}CD38^{int}CD24^{int}CD27^{+}$ cells (Sims *et al*. 2005; Aaltonen *et al*. 2010)

In summary, the human B cell phenotype has permitted the identification of B cell subsets at different stages of maturation. These surface markers have then facilitated the study of the B cell function and its participation in the immune response.

1.3.5 Human B cell surface molecules

Surface molecules	Function	Expressed in	Also found in
BCR	Antigen recognition	All B cells	-
CD20	? Role in B cell activation or regulation; calcium channel	All B cells	-
CD19	B cell activation; forms a co-receptor complex with CD21 and CD81 which delivers signals from BCR complex	All B cells	-
CD1d	Presentation of non-peptide (lipid and glycolipid) antigens to some T cells	Transitional B cells	Thymocytes, DCs and intestinal epithelial cells
CD5	Signalling molecule; binds CD72	Transitional B cells	T cells
CD10	Metalloproteinase; B cell development	Immature B cells, Transitional B cells	Lymphoid progenitors and granulocytes
CD11a	Cell:cell adhesion; binds to ICAM-1, ICAM-2 and ICAM-3	All B cells	Leukocytes
CD21	Receptor for complement fragment CD3d; forms a co-receptor complex with CD19 and CD81 which delivers activating signals in B cells; Epstein-Barr virus receptor	Mature B cells	Follicular DCs
CD22	Regulation of B cell activation; adhesion molecule	All B cells	-
CD23	Low-affinity Fcε receptor, induced by IL-4	Activated B cells	Monocytes, macrophages
CD24	Unknown	Transitional B cells (++) Naïve B cells (+)	Granulocytes
CD25	Binds IL-2 because it is a subunit of the IL-2 receptor	Activated B cells	Activated T cells, regulatory T cells and macrophages
CD27	Binds CD70; can function as a co-stimulator for T and B cells	Memory B cells	Medullary thymocytes, T cells, NKs
CD32	Fc receptor for aggregated IgG; binds C-reactive protein; role in phagocytosis, acts as an inhibitory receptor that terminates activation signals initiated by the BCR	All B cells	Macrophages, Granulocytes, eosinophils and platelets
CD38	NAD glycohydrolase, augments B cell proliferation	Transitional B cells (++) Naïve B cells (+)	Activated T cells, germinal centre B cells and Plasma

			cells
CD40	Binds CD154; role in T cell-dependent B cell, activation, as well as macrophage, DCs, and endothelial cell activation	All B cells	Macrophage, DCs, and endothelial cell
CD44	Binds hyaluronic acid and mediates adhesion of leukocytes	MatureB cells	Leukocytes and erythrocytes
CD62L	Leukocyte-endothelial adhesion; homing of Naïve T cells to peripheral lymph nodes	All B cells	T cell, monocytes, granulocytes and some NKs
CD79a	Required for B cell surface expression of, and signal transduction by, the BCR-antigen complex	All B cells	-
CD79b	Required for B cell surface expression of, and signal transduction by, the BCR-antigen complex	All B cells	-
CD80	Co-stimulator for T lymphocyte activation; ligand for CD28 and CTLA-4	Activated B cells	Macrophage and DCs
CD81	B cell activation; forms a co-receptor complex with CD19 and CD21 which delivers signals that synergise with signals from BCR-antigen complex	All B cells	T cells, NK cells, DCs, thymocytes and endothelium
CD86	Co-stimulator for T cell activation; ligand for CD28 and CD152	Activated B cells	Monocytes, DCs and some T cells
CD138	Heparan sulphate proteoglycan binds collagen type I	Plasma cells	B cells

(Murphy *et al*. 2012)

Table 2: Human B cell surface molecules

1.3.6 The Humoral Response

Antigen recognition

The humoral response begins when the immunoglobulin from the BCR recognises and binds specific antigens with high affinity. Depending on the type of antigen the humoral response can be Thymus Independent (TI), when antigens can induce antibody production in the absence of helper T cells, or Thymus Dependent (TD), when antigens require antigen-specific T cell help to produce antibodies.

Thymus independent response

Antigens, such as bacterial polysaccharides, polymeric proteins and lipopolysaccharides, are capable of stimulating Naïve B cells to induce B cell activation, maturation and differentiation in the absence of T helper cells. TI antigens can be divided in two classes: TI-1 antigens that possess the intrinsic capacity to induce cell division, and TI-2 antigens, which contain no intrinsic B cell-stimulating activity. Whereas TI-1 antigens can activate both immature and mature B cells, TI-2 antigens can activate only mature B cells. LPS is an example of a B cell mitogen and TI-1 antigen, while polysaccharide capsule from bacteria is an example of TI-2 antigen.

Thymus dependent response

Linked recognition by both B cell and T helper cells that respond to the same antigen, is essential in the TD response to induce differentiation of Naïve B cells into Memory or antibody-secreting Plasma cells. Beside peptide/MHC class II-TCR recognition between B and T cells, CD40-CD40L is another important interaction in the correct function of this response. CD40 ligand (CD154) is a T cell effector protein from the TNF family-member that interacts with CD40; CD40 is an activation molecule from the TNF-receptor family member and it is important in the DI response because along with IL-4, CD40 activation induces B cell proliferation, immunoglobulin class switching, and somatic hypermutation. CD30/CD30L, CD28/CD80-CD86, B7-RP/ICOS are other cell-bound proteins participating in the DI responses, and IL-5 and IL-6, are as well cytokines involved in this interaction.

The B cell co-receptor

The B cell-co-receptor complex is formed by three surface proteins: CD19, CD21 and CD81. This complex reduces the threshold for B cell activation *via* BCR, by linking the antigen specific recognition with CD21-mediated complement. CD21 is a complement receptor that binds the C3d fragment. Thus, CD3d-bound pathogens can cross-link with the BCR signalling pathway through this co-receptor complex. After BCR activation, the Src-family tyrosine kinase induces phosphorylation of the CD19 cytoplasmic tails, and after phosphorylation, PI 3-kinases continue with the signalling pathway activating transcription factors such as NFkB, NFAT and AP-1, to finally induce cell proliferation and differentiation.

Antigen specific B cell – T cell encounter

Following the BCR recognition and activation, circulating Naïve B cells migrate into peripheral lymph nodes through endothelial venules, and enter in the T cell areas to find an antigen-specific T cell that shares the same antigen-specificity. The chance of a random encounter between these antigen-specific cells is between 1 in 10^8 to 1 in 10^{12} (Murphy *et al*. 2012). To facilitate the encounter, once the B cell has bound the antigenic protein, the cell expresses adhesion molecules (LFA-1), and chemokine receptors (CCR7, CCL19, CCL21); both signals trap the B cell in the T cell zone. After contact, antigen-specific B and T cells migrate to the border of the red pulp, where the B cells proliferate to form a primary focus, or migrate into a primary lymphoid follicle to form a germinal centre.

Antibody-producing cell

In the primary focus, proliferation of antigen specific T and B cell can last several days after encounter. Some of the proliferating B cells differentiate into antibody-synthesising Plasmablasts. After a few more days, these stop dividing and either die or differentiate into Plasma cells. Some of these short-lived Plasma cells will remain in the lymphoid organ, while the majority will migrate to the bone marrow.

The rapid antibody secretion pathway described above, is an immediate protective response for the infected individual. However, there is a more effective response that includes somatic hypermutation which occurs when B cells migrate into a primary lymphoid follicle and forms a germinal centre with T helper and follicular DCs. Germinal centre B cells differentiate into long-lived Plasma cells, able to secrete all antibody classes, or long-lived descendant class-switch Memory B cells that inherit the genetic changes that occurred in the germinal centre. These Memory B cells express the class-switch corresponding immunoglobulin in their surface, but they are incapable of secreting antibodies.

Antibodies

Antibodies, secreted by Plasma cells, are the main players in the humoral effector response. They are proteins from the globulin family that exhibit diverse structures, locations and function. Two domains, variable and constant, form the basic immunoglobulin structure, and both domains are located in the heavy and light chain of the antibody.

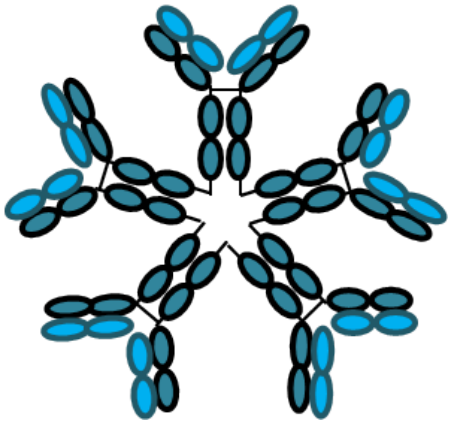
The specificity of an antibody response is determined by the antigen-binding site, located in the variable domain, but the final effector action will be determinated by the isotype of the heavy chain from the constant domain; both regions experience changes in order to improve antibody function and affinity.

Class-switch recombination involves only the constant region of the antibody, while somatic hypermutation and gene conversion affects mainly the variable region. In the former process, an alternative heavy-chain replaces the Cμ heavy-chain C region, and the latter process alters the affinity of the antibody for the antigen. IgM, IgD, IgG, IgA and IgE are the five-immunoglobulin classes described in humans and their names are derived from the corresponding heavy chain (μ, δ, γ, α and ε). Moreover, IgG can be divided in four sub-classes, IgG1, IgG2, IgG3 and IgG4 and IgA can be divided in two sub-classes, IgA1 and IgA2.

All Naïve B cell express cell-surface IgM and IgD. Even when IgM is the first antibody secreted, IgG and IgA are the predominant antibodies found in circulation, implying the importance of the class switch process. The mechanism of class switching

between regions occurs when the rearranged V region is located in front of the corresponding C region. The selection of a C region is not random and is regulated by specific cytokines secreted by T cells, or other cells, during the humoral response. IL-4 preferentially induces IgG1 and IgE, TGF-β induces IgG2b and IgA, IFN-γ induces IgG2a and IgG3, and IL-5 augments IgA production. Cytokines induce class switching in part by stimulating the production of RNA transcripts from the switch recombination sites in the heavy-chain domain. CD40L-CD40 interaction is another signal required for class switching. Several cases of hyper IgM syndrome has been reported, in which CD40L-deficient individuals exhibit abnormally high levels of IgM, with low levels of the other classes (Agematsu *et al*. 1998).

IgM



Structure: IgM normally exists as a pentamer, but it can also exist as a monomer. In the pentameric form, all heavy and light chains are identical. IgM has an extra domain on the μ chain (C_{H4}) and it has another protein covalently bound *via* an S-S bond called the J chain. This chain plays a role in the polymerisation of the molecule into a pentamer.

Location: B cell surface and serum.

Secretion: IgM is the third most common serum Ig.

Function: IgM is the first Ig produced by the foetus, and the first Ig expressed by Naïve B cells before antigen encounter. As a consequence of its pentameric structure, IgM is also a good complement fixing and a good agglutinating Ig. Furthermore, IgM antibodies are very proficient in clumping microorganisms for eventual elimination from the body.

IgD

Structure: Exists only as a monomer.

Location: Primarily found on B cell surfaces.

Secretion: Low levels in serum.

Function: B cell Receptor

IgG

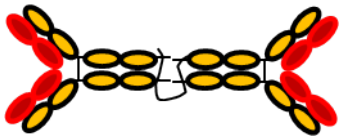
Structure: The structures of the IgG subclasses are all monomers. The subclasses differ in the number of disulphide bonds and length of the hinge region.

Location: B cell surface, serum and extra vascular spaces.

Secretion: IgG is the major Ig in serum (75% of serum), and the major Ig in extra vascular spaces.

Function: IgGs fix complement and can then be recognised by macrophages, monocytes, PMNs and some lymphocytes because of the Fc receptors exhibited by these cells. Although not all subclasses bind Fc receptors equally well, as IgG2 and IgG4 are not able to bind Fc receptors, the binding of other subclasses such as IgG1 and IgG3 improve the internalisation of the antigen in PMNs, monocytes and macrophages (Brooks *et al*. 1989). The term opsonin is used to describe substances that enhance phagocytosis; IgG is a good opsonin.

IgA



Structure: IgA is a monomer in serum, and a dimer with an associated J chain in secretions. When IgA is found in secretions, another protein called the secretory piece or T piece is associated with the antibody. The secretory piece is made in epithelial cells, and is added to the IgA as it passes into the secretions.

Location: B cell surface, secretion and serum.

Secretion: IgA is the main Ig in secretion as tears, saliva, colostrums and mucus. It is also the 2nd most common serum Ig.

Function: IgA is important in local mucosal immunity. IgA can bind to some cells – PMNs and some lymphocytes.

IgE

Structure: IgE exists as a monomer and has an extra domain in the constant region.

Location: B cell surface (the least Ig abundant isotype in serum)

Secretion: Allergic reaction and parasitic infections.

Function: IgE binds very tightly to Fc receptors on basophils, mast cells and eosinophils. IgE is involved in allergic reactions as a consequence of binding to basophils and mast cells, and is also involved in parasitic reactions as a consequence of binding Fc receptors on eosinophils.

The importance of the B cells in antibody production is undeniable, particularly due to the different functions displayed by these Igs. In spite of that, B cells can also function as professional APCs and cytokine-secreting cells. These alternative functions can participate in both rejection and tolerance depending on the B cell subset, immune environment, activation, number of antigens, and so on.

1.3.7 B cells as APCs

As mentioned previously, B cells are professional APCs because they present antigenic peptides in the context of MHC class II.

In addition, they exert several APC properties:

- They up-regulate the expression of chemokines that allow expression of other cytokines in T cells, which then allow the migration of T cells to secondary lymphoid organs.
- BCR-mediated endocytosis allows them to concentrate small amounts of specific antigens.
- The HLA-DO expression on B cells favours presentation of peptides derived from antigens internalised through the BCR (Rodriguez-Pinto 2005).

In terms of the regulatory role of B cells that act as APCs, CD40-activated B cells expressed high levels of MHC class I and II, and high levels of the co-stimulatory molecules CD80 and CD86 (Tu *et al*. 2008). These activated B cells can induce alloantigen-specific $CD4^{high}CD25^{+}$ Treg (Tu *et al*. 2008) and alloantigen-specific $CD8^{high}$ Tregs (Zheng *et al*. 2009); both T cell subsets express $FoxP_3$, CTLA-4 and CD62L. The same group demonstrated that CD40-activated B cells are more potent in generating large number of antigen-specific Tregs compared to immature DCs (Zheng *et al*. 2010). Another group demonstrated that CD40-activated B cells induce T-cell chemotaxis by expressing CD62L, CCR7/CXCR4, and LFA-1 (von Bergwelt-Baildon *et al*. 2006).

The interaction between the T and B cell requires communication between proteins from both cells and although molecule interaction between B and T cells are described (Clark *et al*. 1994), it is still unknown how B cells can command a pro-inflammatory or an anti-inflammatory response on T cells.

1.3.8 Cytokine-Producing B cells

Although B cells are not traditionally known as cytokine-secreting cells, they can produce several cytokines depending on the activation used. These cytokines can exert a pro-inflammatory or an anti-inflammatory role in the immune response, depending on the function and the type of cytokine secreted.

O'Garra *et al*. examined the B cell ability to produce cytokines in murine Ly-1^+ B lymphomas and in normal murine peritoneal B cells. Results showed that all the B cells studied were capable of expressing IL-6, TNF-α, TNF-β and IL-10, after LPS activation. B lymphomas also produced different cytokines to normal B cells such as IL-3, IL-4 and GM-CSF (O'Garra *et al*. 1990).

Kouskoff *et al*. studied cytokine production after BCR activation in B cells from a 3-83 transgenic mouse. They found no changes in IL-4, IL-5, IL-9, IL-10, IL-13, and IL-15 mRNAs levels after antigen activation. Despite that, after 6 hours of stimulation with P31, a clear increase in mRNAs was observed for IL-6, IL-2, and IFN-γ (Kouskoff *et al*. 1998).

Kindler *et al*. identified IL-2 production by normal human B cells. They investigated various B cell-activating conditions, and showed that B cells secreted IL-2 when first activated by CD40, over 24 hours before PMA and ionomycin activation. Lower IL-2 expression was detected when cross-linking anti-IgM antibodies were used instead of PMA plus ionomycin. Their results showed that the production of IL-2 by normal B lymphocytes occurs as a late event relative to their activation and proliferation (Kindler *et al*. 1995).

IL-4 is known as an immunomodulatory cytokine secreted by relatively few cell types, and plays an important role in promoting the production of the IgE antibody. IL-4 was found in supernatants of Epstein-Barr virus (EBV)-positive B cell lines from PBMCs of patients with atopic dermatitis (AD). This report was the first evidence of IL-4 production by an EBV-transformed B cell line (Ohnishi *et al*. 1997). IL-12 was also found in supernatant from human EBV-transformed B cell lines. However, phagocytic cells rather than B cells appear to be the most important physiological producers of IL-12 (Sartori *et al*. 1997).

Harris *et al*. identified in humans, two populations of "effector" B cells, Be1 and Be2, which produced distinct patterns of cytokines depending on the cytokine environment in which the cells were stimulated during their primary encounter with antigen and T cells. Be1 cells regulated the differentiation of naïve $CD4^+$ T cells into Th1 cells *via* IFN-γ and IL-12 secretion, while Be2 induced Th2 differentiation through

IL-4, IL-6 and IL-10 production. In conclusion cytokines produced by B cells were able to regulate T cell polarisation (Harris 2000).

Finally, Duddy's group reported that different B cell subsets secreted different type of cytokines in human cells. Firstly, cytokine expression was studied in B cells after BCR/CD40 activation. Results showed that B cells appropriately stimulated by sequential BCR and CD40 stimulation, proliferated and secreted TNF-α, LT, and IL-6. In contrast, CD40 stimulation alone, induced negligible pro-inflammatory cytokines, but significant production of IL-10 (Duddy *et al*. 2004). Secondly, the same group evaluated similar activation in B cells subsets. Results revealed that Memory B cells were the main LT and TNF-α–secreting cells after BCR/CD40 stimulation, whereas Naïve B cells secreted mainly IL-10 when CD40 activation was used alone (Duddy *et al*. 2007). More recently, $CD24^{hi}CD38^{hi}$ Transitional B cells, but not $CD24^{int}CD38^{int}$ Naïve or $CD24^{hi}CD38^{-}$ Mature B cells, were capable of producing IL-10 after CD40L stimulation (Blair *et al*. 2010).

1.3.9 B cells as a regulatory cell in mice

In 1996, Wolf *et al*. demonstrated for the first time the role of the B cell as a regulatory cell in an animal model of Experimental Autoimmune Encephalomyelitis (EAE), an autoimmune central nervous system disease mediated by $CD4^{+}$ T cells. They studied the participation of B cells in the induction and progression of EAE, and found that B cell-deficient mice exhibited a greater response in the disease onset and severity, compared to controls. Their data suggested that B cells did not play a role in the activation of encephalitogenic T cells, but played a role in the immune regulation of EAE through T cell deviation from Th1 to Th2 cytokines, possibly by altering peptide dose, complexity, or co-stimulation expression (Wolf *et al*. 1996).

One year later, Mizoguchi *et al*. suggested that although B cells were not required for the initiation of colitis, they suppressed colitis, presumably by affecting the clearance of apoptotic cells (Mizoguchi *et al*. 1997). Although this may be true, years later, IL-10 production was proposed as the main regulatory mechanism used by B cells in EAE (Fillatreau *et al*. 2002) and arthritis (Mauri *et al*. 2003) in mice. The main IL-10-producing cells were characterised as $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}CD1d^{hi}$ cells and they were found after CD40 activation within the immature transitional two-marginal

zone precursor B cell subset (T2-MZP) in arthritis (Evans *et al*. 2007) and lupus (Blair *et al*. 2009) in mouse models. In addition, another study demonstrated that B cells immune-regulated autoimmune disease in a murine lupus model. CD19 deficient mice exhibited early pathologic manifestations of nephritis and reduced survival, compared to wild type mice (Watanabe *et al*. 2010).

A regulatory role of the B cells was observed in diabetes. LPS-activated B cells, but not control B cells, expressed Fas ligand and secreted TGF-β. In addition, transfusion of activated B cells, but not control B cells, into pre-diabetic NOD mice, inhibited spontaneous Th1 autoimmunity, but did not promote Th2 responses to β cell autoantigens. These data suggested that activated B cells down-regulated pathogenic Th1 response *via* TGF-β production (Tian *et al*. 2001).

Other studies revealed a protective role of B cells in inflammatory bowel disease. Mesenteric lymph node B cells protected mice from colitis induced by $Gai2^{-/-}$ $CD4^{+}$ T and $CD4^{+}CD45RB^{hi}$ T cells. B cells were associated with the induction of regulatory T cells in the mucosal immune homeostasis (Wei *et al*. 2005).

Several mechanisms have been described for regulatory B cells in mice, especially in autoimmune diseases. Cell contact and cytokine secretion seem to be involved in B cell mediated-suppression. Knowing this, groups moved to humans to verify if these cells were also a playing an important role in different diseases.

1.3.10 B cells as a regulatory cell in humans

The identification of regulatory B cells in humans is not fully established and the first studies were based on the phenotype described in mice. The characterisation of human regulatory B cells present some similarities and some differences to the IL-10-producing B cell subset in mice; for example, human B cells secreted IL-10 after CD40 activation, the same way that B cells from mice did (Duddy *et al*. 2004; Jamin *et al*. 2008); conversely, the main location of mouse B cells was from the spleen, whereas in humans, regulatory B cells were mainly located in the bone marrow and peripheral blood.

To identify this subset, Sims and colleagues studied the phenotype of B cells during different stages of maturation. They revealed that the transitional population,

located in peripheral blood, was very similar to the phenotype exhibited by immature B cells from the bone marrow (Sims *et al*. 2005; Palanichamy *et al*. 2009).

Interestingly, before the complete description of the transitional B cell phenotype in humans, these cells were related to immunodeficiency, as patients with severe immunodeficiency exhibited an increment of circulating immature/transitional B cells with a notorious reduction of circulating Memory B cells (Martinez-Maza *et al*. 1987; De Milito *et al*. 2001). Expansion of functionally immature Transitional B cells was observed in human-immunodeficient states, characterised by impaired humoral immunity (Cuss *et al*. 2006). A novel form of primary immune disorder was also characterised with circulating B cells unable to respond to CpG stimulation. These cells exhibited the $CD24^{hi}CD38^{hi}CD27^{-}$ Transitional phenotype, and B cells from these patients did not proliferate and failed to secrete immunoglobulins after *in vitro* CpG stimulation (Plebani *et al*. 2007).

Continuing with the characterisation, Transitional B cells exhibited low viability in humans. IL-4, CD40L and Mesenchymal Stem Cells (MSC) were able to increase the viability of these cells in culture (Sims *et al*. 2005; Cuss *et al*. 2006; Tabera *et al*. 2008; Lee *et al*. 2009), and to suppress B-cell terminal differentiation (Asari *et al*. 2009).

Finally, as was mentioned previously, CD40–activated B cells $IgD^{+}IgM^{+}CD86^{+}$ were able to inhibit the immune response, increasing the CD4 and the CD8 Treg population, demonstrating a regulatory role as an APC (Tu *et al*. 2008; Zheng *et al*. 2009). In addition, $CD19^{+}CD24^{hi}CD38^{hi}$ regulatory B cells were able to inhibit $CD4^{+}$ T cells pro-inflammatory cytokine production by cell-cell contact and IL-10 production, demonstrating a regulatory role as a cytokine producing cell (Blair *et al*. 2010). Interestingly, $CD19^{+}CD24^{hi}CD38^{hi}$ regulatory B cells from patients with systemic lupus erythematosus were impervious to CD40 stimulation, losing the regulatory properties exhibited in healthy individuals (Blair *et al*. 2010). Kidney transplant recipients that developed spontaneous tolerance after withdrawing immunosuppressive therapy exhibited an increased number of B cells and a B cell gene signature in peripheral blood, suggesting an unknown regulatory role in graft maintenance (Newell *et al*. 2010; Pallier *et al*. 2010; Sagoo *et al*. 2010). With this background a number of questions remain unanswered, particularly in transplantation tolerance. This thesis was proposed to test the following hypothesis and aims.

2 Hypothesis and Aims

2.1 Transitional B cells are expanded in peripheral blood samples from tolerant kidney transplant recipients.

Aims

- Measure the percentage of the B cell subsets in kidney transplant patients and healthy controls to establish the differences between B cell subsets between groups.

2.2 Transitional B cells from tolerant recipients modulate an anti-inflammatory response and therefore participate in the state of tolerance.

Aims

- Measure the expression of B cell activation markers and cytokine production after CD40L and CpG activation in B cells from kidney transplant patients and healthy controls to establish differences between B cell regulatory responses between groups.

2.3 Transitional B cells are able to modulate active allogeneic responses and therefore play a key role in inducing donor-specific tolerance.

Aims

- Measure the percentage of donor-specific B cells and the $CD4^+$ T cell response after donor-antigen presentation by B cells from kidney transplant patients.

2.4 Identification of B cell-related genes and measurement of their expression in kidney transplant patients would allow a better prediction of the outcome of a renal transplant.

Aims

- Analysis of genes tolerance-related in kidney transplant patients and healthy controls to find a relationship between the function of the genes on the B cell and the role than the B cells could be playing to induce tolerance.

2.5 Transitional B cells exhibit an anti-inflammatory response compared to Naïve and Memory B cells.

Aims

- Perform co-cultures between allogeneic $CD4^+$ T cells and the different B cell subsets in order to study the participation of Memory, Naïve and Transitional B cells in the induction of T cell proliferation, activation and cytokine secretion.

3 Materials and Methods

3.1 Phenotypical and functional characterisation of B cell subsets in kidney transplant patients.

3.1.1 Study purpose

The purpose of this thesis was focused on identifying the role of Transitional B cells in renal transplant tolerance. Samples facilitated from the Genetic Analysis & Monitoring of Biomarker of Immunological Tolerance (GAMBIT) study were used in this research project.

3.1.2 Study design

The GAMBIT study is cross-sectional observational study established to validate a set of biomarkers of tolerance in kidney transplantation. Of the patients included in GAMBIT, the following ones have been used in this project:

a) **Tolerant:** Functionally stable kidney transplant recipients (serum creatinine CRT < 160umol/l and < 10% rise in the last 12 months) despite having stopped all their immunosuppression for longer than one year

b) **Stable:** Functionally stable kidney transplant recipients (serum creatinine CRT < 160umol/l and < 10% rise in the last 12 months) maintained with immunosuppressive agents. Recipients were age and gender matched to tolerant patients.

c) **Stable in Monotherapy:** Functionally stable kidney transplant recipients (serum creatinine CRT < 160umol/l and < 10% rise in the last 12 months) maintained only with one immunosuppressive agent at a low dose.

d) **Chronic rejector:** Kidney transplant recipients with graft dysfunction and a recent biopsy showing signs of immunological driven chronic rejection.

e) **Healthy Control:** Healthy volunteers, age and gender matched to tolerant patients.

Patients that had been identified as tolerant for a period of time, but showed graft dysfunction eventually, have been identified as "**Loss Tolerance**". Although these patients were also studied, their results have not been included in the statistical analysis used by the former patient groups, as only three patients were recruited.

f) **Loss Tolerance:** Previous tolerant kidney transplant recipients that lost their state of tolerance and started to present signs of graft dysfunction.

Peripheral blood and serum samples were taken after informed consent was obtained. The study was approved by the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee 09/H0713/12. All samples were processed and analysed in a blinded fashion.

3.1.3 Recruitment sites

Recruitment sites from the UK:

- **Guy's Hospital**, London.
- **The Evelina Children's Hospital**, London.
- **Great Ormond St Children Hospital**, London.
- **Royal Free Hospital**, London.
- **St George's Hospital**, London.
- **King's College Hospital**, London.
- **Salford Royal Hospital**, Salford.
- **Cardiff and Vale University Health Board**, Cardiff.
- **Queen Alexandra Hospital**, Portsmouth.
- **Hull Royal Infirmary**, Hull.
- **St James's University Hospital**, Leeds.
- **Leicester General Hospital**, Leicester.
- **Manchester Royal Infirmary**, Manchester.
- **Northern General Hospital**, Sheffield.
- **Kent and Canterbury Hospital**, Kent.

- **Glasgow General Hospital**, Glasgow.

Recruitment site from Switzerland:

- **INSELSPITAL, Universitätsspital**, Bern.

Recruitment site from Czech Replublic:

- **Transplantační laboratoř IKEM**, Prague.

Recruitment site from Spain:

- **Hospital Universitari Vall d'Hebrón**, Barcelona.

3.1.4 Inclusion and exclusion criteria

Renal transplant patients from all groups with haemoglobin >10 mg/dL were included in the study. Patients with acute infection, Human Immunodeficiency Virus, active Hepatitis C Virus or Hepatitis B Virus were excluded from the study.

3.1.5 Data collection

Data collection included non-clinical and clinical parameters, transplant data and immunosuppressive therapy used; all these parameters have been described in Table 3. Data was collected from patient medical records and entered into an encrypted SQL Server Database, uniquely designed for the GAMBIT study.

	Healthy Controls	Tolerant	Stable	Monotheraphy	Chronic Rejector
Age (years)	48.0 (23-72)	50.6 (22-77)	52.9 (22-84)	54.8 (43-80)	41.0 (18-72)
Number of patients	12	16	8	13	20
Sex					
-Male	10	13	6	7	15
-Female	2	3	2	6	5
Recipient age at Transplant		32.1 (11-56)	33.5 (14-62)	34.7 (14-48)	33.5 (11-70)
Donor (Living/Cadaveric)		(8/8)	(4/4)	(4/9)	(9/11)
Time of transplantation		18.5 (3-34)	18.8 (8-28)	20.1 (6-32)	7.9 (1-26)
HLA mismatches					
A		10/16	5/8	7/13	16/20
B		7/16	5/8	9/13	17/20
DR		7/16	6/8	2/13	16/20
Immunosuppressive Drugs					
Cyclosporine/Tacrolimus		0/16	5/8	8/13	20/20
Azathioprine		0/16	5/8	0/13	1/20
Prednisone		0/16	6/8	6/13	15/20
MMF		0/16	2/8	0/13	14/20
Clinical Parameters					
Creatinine		114.8	140.5	107.1	222.2
eGFR		64.8	55.3	65.1	33.2
White Blood count x 10^9		6.9	7.6	7.9	7.3
Lymphocytes x 10^9		1.9	1.7	2.0	1.1
B cell Count x10^9		0.32 ± 0.21	0.06 ± 0.04	0.18 ± 0.22	0.12 ± 0.11
ANOVA Tol vs all			** p = 0.0039	NS	** p = 0.0042
Transitional B cell count x10^9		0.029 ± 0.026	0.001 ± 0.002	0.009 ± 0.008	0.001 ± 0.001
ANOVA Tol vs all			*** p = 0.0002	** p = 0.0026	**** p < 0.0001

Table 3: Clinical and demographic data of kidney transplant patients.

3.1.6 Blood and serum samples collection from kidney transplant patients and healthy controls

Blood and serum samples collection from kidney transplant patients and healthy controls was performed by the team of research nurses from the GAMBIT study. All volunteers were maintained seated during 15 minutes prior to phlebotomy in order to eliminate possible interferences of blood distribution due to different posture. After this interval, a vein was located and blood samples were drawn using winged infusion set (butterfly needle, Greiner bio-one) directly into vacuum EDTA (Ethylenediaminetetraacetic acid) purple tubes (BD Vacutainer) and serum red tubes (BD Vacutainer). Blood sample tubes were properly labelled and delivered into the laboratory for further processing and storage. Three blood samples were collected between three to six months. 60ml of blood was collected in the two first samples each and 150ml of blood was collected in the last sample.

3.1.7 Blood and serum samples processing and storage from kidney transplant patients and healthy controls

PBMCs from patients and healthy controls were isolated from peripheral blood using Ficoll-Hypaque (PAA) density gradient centrifugation. Cells were washed, resuspended in 10% DMSO (Sigma-Aldrich) - AB human serum (BioWest) and frozen immediately at -80°C. After 24 hours, cells were stored in liquid nitrogen and kept until use. Serum samples were obtained after centrifugation of coagulated peripheral blood samples (2000RPM / 20min / room temperature), stored at -80°C and kept until use.

3.1.8 Spleen and lymph nodes samples

Spleen and lymph nodes samples were obtained from cadaveric donors from spare material sent for cross monitoring to tissue typing laboratory. Samples were cut in pieces and mashed in gentleMAC C Tubes (Myltenyi Biotech) for 10 minutes. Cells were filtered twice, washed, resuspended in 10% DMSO (Sigma-Aldrich) - AB human serum (BioWest) and frozen immediately at -80°C. After 24 hours cells were stored in liquid nitrogen and kept until use.

3.1.9 B cell phenotypic analysis on PBMCs from kidney transplant patient and healthy controls

PBMCs and cells obtained from spleen and lymph nodes were thawed from liquid nitrogen the same day of the staining. Cells were counted and $3x10^6$ PBMCs, and $1x10^6$ cells from lymphoid tissues, were placed into 5ml polystyrene tube. To assess cell death, cells were stained with Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 10 minutes at 4°C. Then, cells were washed and stained with anti-CD19-AlexaFluor780 (eBioscience), anti-CD20-AlexaFluor700 (eBioscience), anti-CD27-APC (eBioscience), anti-IgD-PE (BD), anti-IgM-PerCP-Cy5.5 (BD), anti-CD24-FITC (eBioscience) and anti-CD38-PECy7 for 30 minutes at 4°C. Stained cells were washed, resuspended in 300ul of PBS and acquired on LSRFortessa (BD). LSRFortessa stability for patient samples was controlled with BD Cytometer Setup & Tracking Beads (CS&T) and application settings. Data was analysed using DIVA software (BD).

3.1.10 B cell death and apoptosis measurement

Standardisation of B cell death and apoptosis was performed in PBMCs, B cells and B cell subsets. $1x10^6$ cells were stained with Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 10 minutes at 4°C to assess cell death. Then, cells were washed and stained with anti-BAFFr-FITC (eBioscience) for 30 minutes at 4°C. Cells were washed, resuspended in 500ul of AnnexinV 1X Binding Buffer (eBioscience) and stained with AnnexinV-eFluor450 (eBioscience) for 10 minutes at 4°C; both staining were performed to assess apoptosis. Finally, cells were resuspended in 300ul of AnnexinV 1X Binding Buffer (eBioscience) and acquired on LSRFortessa (BD). Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

Apoptotic B cells from kidney transplant patients were measured by surface staining with Live/Dead dye (Invitrogen) and anti-BAFFr-FITC (eBioscience). Finally, the viability of the B cell subsets from healthy volunteers was evaluated in the presence of tacrolimus (Sigma-Aldrich), mycophenolate mofetil (MMF) (CellCept, Roche) and steroids (SOLU-CORTEF, Pharmacia) *in vitro*. Cells were incubated in the presence of tacrolimus 0-25ng/ml, MMF 0-25ug/ml or steroids 0-500ng/ml overnight and Live/Dead dye and BAFFr staining were performed the next day to evaluate cell death and apoptosis. Cells were acquired on LSRFortessa (BD) and a minimum of 10.000 B

cell events were collected per sample. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.1.11 CD20 and CD19 Median Fluorescence Intensity (MFI) analysis

MFI of CD20 and CD19 (anti-CD20-AlexaFluor700 and anti-CD19-AlexaFluor780 (eBioscience)) was obtained from statistical data extracted from the B cell panel described in Methods 3.1.9. B cell markers co-localisation was performed in PBMCs stained with anti-CD19-AlexaFluor780 (eBioscience), anti-CD20-AlexaFluor700 (eBioscience), anti-CD27-APC (eBioscience), anti-IgD-PE (BD), anti-IgM-PerCP-Cy5.5 (BD), anti-CD24-FITC (eBioscience) and anti-CD38-PECy7 for 30 minutes at 4°C. Data was acquired on a FlowSight system equipped with four lasers, running with Inspire software, and analysed with IDEAS 3.0 software (all from AMNIS Corp, Seattle, WA, USA). Delta-centroid, polarisation and co-localisation of B cell markers were assessed with specific features/masks within IDEAS 3.0. A minimum of 100.000 B cell events were acquired per sample.

3.1.12 B cell functional assays in response to CD40L and CpG activation measured in kidney transplant patient and healthy control's PBMCs

PBMCs and mouse L fibroblast (L cells) were thawed 24 hours before all functional assays. CD40L-transfected and non-transfected L cells were thawed, washed, resuspended in 1ml of complete media and X-ray irradiated for 30 minutes, receiving a total dose of 9045 cGy (RADS) (The Gammacell 1000, Elite). $0.5x10^5$ CD40L-transfected and non-transfected L cells were cultured in 48-well plates (Nunc, Langenselbold, Germany) overnight. Expression of CD40L in transfected and non-transfected L cells was measured by surface staining using an anti-CD40L-PE (eBioscience) antibody. PBMCs from kidney transplant patients and healthy controls were thawed, washed and rested overnight in RPMI-1640 (Sigma) supplemented with 2mM L-Glutamine (Gibco, Invitrogen), 100U/mL penicillin, 100mg/mL streptomycin (Gibco, Invitrogen) and 10% of Foetal Calf Serum (FCS) (SeraQ, Sussex, UK) in 24-well plates (Nunc, Langenselbold, Germany) at 37°C 5% CO_2. The day of the experiment, $1x10^6$ PBMCs were cultured with and without $0.5x10^5$ plate bound CD40L-transfected L cells, $0.5x10^5$ plate bound non-transfected L cells and CpG (1uM) (ODN

2006, InvivoGen) for 72hours. After 3 days, activation markers CD86-FITC (eBioscience), CD40-APC (eBioscience) and CD25-PE (eBioscience) were measured by surface staining in total, Memory and $CD27^-$ B cells after CD40 activation. TLR-9 expression was measured in CpG-activated and non-activated B cells by intracellular staining using an anti-TLR-9-PE (eBioscience) antibody. Cytokine production (IL-10, IFN-γ and TNF-α) from CD40- and CpG-activated B cells was measured using intracellular staining and ELISA.

3.1.13 Intracellular Staining

Cells were activated with Phorbol 12-myristate 13-acetate (PMA) (50ng/mL, SIGMA), ionomicyn (1ug/mL, SIGMA), GolgiStop (1X, BD) and Brefeldin A (1X, BD) for 5 hours in new culture media at 37°C 5% CO_2. Cells were washed with PBS and stained with Live/Dead reagent for 10 minutes at 4°C. Cells were washed again and stained with anti-CD20-AlexaFluor780 (eBioscience) and anti-CD3-Pacific Blue for 30 minutes at 4°C. Cells were then fixed and permeabilised with $FoxP_3$ Fix and Perm Kit (BD) before the intracellular staining with anti-IL-10-PE (BD), anti-IFN-γ-APC (eBioscience) and anti-TNF-α-PECy7 (eBioscience) for 30 minutes at 4°C. Samples were acquired on LSRFortessa (BD) within 24 hours and data was analysed using DIVA software (BD).

3.1.14 ELISA

Enzyme-linked immunosorbet assay (ELISA) (R&D) (eBioscience) was performed according to manufacturer's instructions. Briefly, 96-well plates (Nunc™, Maxisorb, Thermo Scientific) were coated and incubated overnight with 100ul/well of mouse anti-human IL-10 (eBioscience), mouse anti-human IFN-γ (R&D) and mouse anti-human TNF-α (R&D) at working concentrations. Plates were washed three times with 0.05% Tween 20 (Sigma) in PBS and blocked for 1 hour at room temperature with 300ul/ml freshly made filtered 1% bovine serum albumin (BSA) in PBS (w/v). Plates were washed and 100ul/well of standards and supernatant were added in triplicate for 2 hours at room temperature. Standard curves were prepared by serial two-fold dilutions from the maximum concentration recommended by the manufacturer (300pg/ml for IL-10, and 1000pg/ml for IFN-γ and TNF-α). 100ul/well of detection antibodies were added at the working concentrations for 2 hours at room temperature. Plates were washed and 100ul/well of streptavidin-HRP (horseradish peroxidase) was added for 20 minutes at

room temperature. After final washes, plates were incubated with 100ul/well of tetramethylbenzidine (TMB) (eBioscience) for 10 minutes at room temperature and reaction was stopped adding 50ul/well of 2N sulphuric acid (H_2SO_4). Optical density at 405nm was measured using Sunrise ELISA Reader (TECAN, UK). The concentration of each cytokine was calculated from standard curves using Magellan data analysis Software.

3.1.15 CD40 and CpG activation in sorted B cell subsets

CD40 and CpG activation functional assays in sorted B cell subsets were performed to analyse the IL-10 activation pathway in Memory, Naïve and Transitional B cells. Peripheral blood (cones) from healthy volunteers was used to obtain B cell subsets. Blood was incubated with Rosettsep Human B cell enrichment cocktail (STEM CELL) for 20 minutes at room temperature prior Ficoll-Hypaque density gradient centrifugation. After gradient separation, isolated B cells were obtained from the mononuclear layer (purity >70%). Cell viability was determined using trypan-blue exclusion. Cells were stained with anti-CD20-Pacific Blue (eBioscience), anti-CD27-APC (eBioscience), anti-CD24-PerCP-Cy5.5 (BD), anti-CD38-PECy7 (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 30 minutes 4°C. B cell subsets were sorted using ARIA II (BD). Memory B cells were selected as $CD20^{+}CD27^{+}$ (purity >95%), Naïve B cells were selected as $CD20^{+}CD27^{-}CD24^{+}CD38^{+}$ (purity >95%) and Transitional B cells were selected as $CD20^{+}CD27^{-}CD24^{hi}CD38^{hi}$ (purity >95%) (Table 4). $0.5x10^{5}$ B cells subsets were cultured in complete media supplemented with IL-2 (100ng/ml, R&D) with and without $1.0x10^{4}$ CD40L-transfected L cells, $1.0x10^{4}$ non-transfected L cells or CpG (1uM) (ODN 2006, InvivoGen) for 72 hours at 37°C 5% CO_2. After 3 days, IL-10 and TNF-α were measured using intracellular staining (Methods 3.1.13). and ELISA (Methods 3.1.14).

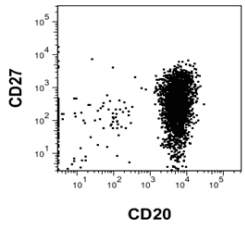
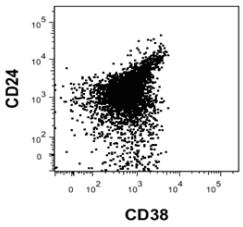
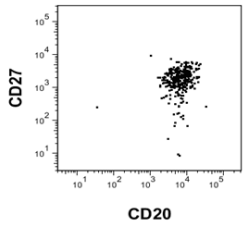
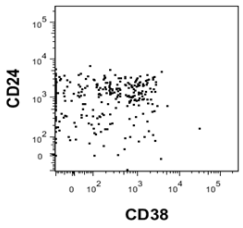
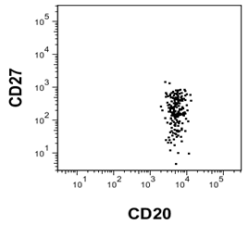
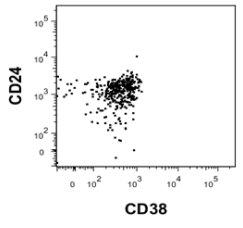
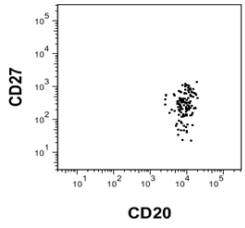
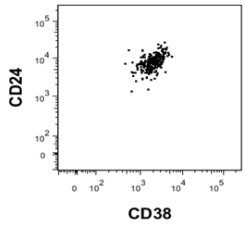
Sample	% Pre-sort	% Post-sort	Plot CD20/CD27	Plot CD24/CD38
Pre-sorted B cells	-	-		
Memory B cells	15-25%	>95%		
Naïve B cells	65-75%	>95%		
Transitional B cells	5-15%	>95%		

Table 4: B cell sort

B cells were pre-enriched using CD20 Rosettsep. Isolated B cells were stained and Memory ($CD20^{+}CD27^{+}$), Naïve ($CD20^{+}CD27^{-}CD24^{+}CD38^{+}$) and Transitional ($CD20^{+}CD27^{-}CD24^{hi}CD38^{hi}$) B cells were sorted using ARIA II. Purity was checked using flow cytometry. B cell subsets presented >95% of purity.

3.2 BCR signalling pathway activation and donor-specific response in kidney transplant patients.

3.2.1 Measurement of phosphorylated proteins after BCR activation

Phosphorylation of BTK, BLNK and ERK was assessed by phospho-flow (Picture 6). Isolated B cells from healthy volunteers were thawed and rested overnight in RPMI-1640 (Sigma) supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2. Next day, $1x10^6$ cells were placed in 5ml polystyrene tubes and activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) (Southern Biotech) antibodies and PMA (0.1uM, SIGMA) for 0, 5 and 10 minutes at 37°C. Cells were fixed with BD Cytofix buffer (BD) for 10 minutes at 37°C. For BTK and ERK staining, cells were permeabilised with BD Phosflow Perm Buffer III (BD) for 30 minutes on ice, and then stained with anti-ERK1/2 (pT202/pY204)-AlexaFluor488 (BD) and anti-BTK (pY551/ltk pY511)-PE (BD) for 30 minutes at 4°C. For BLNK staining, cells were permeabilised with BD Phosflow Perm/Wash Buffer I (BD) for 30 minutes at room temperature, and then stained with anti-BLNK (pY84)-AlexaFluor647 (BD) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.2.2 Activation curve

In order to identify the optimal activation period, isolated B cells from healthy volunteers were activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) antibodies (Southern Biotech) and PMA (0.1uM, SIGMA) for 0, 10 and 30 minutes at 37°C. In order to test the effect of co-activation with CD40L, isolated B cells from healthy volunteers were activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) (Southern Biotech) with and without $0.5x10^5$ CD40L-trasnfected L cells for 10 minutes at 37°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

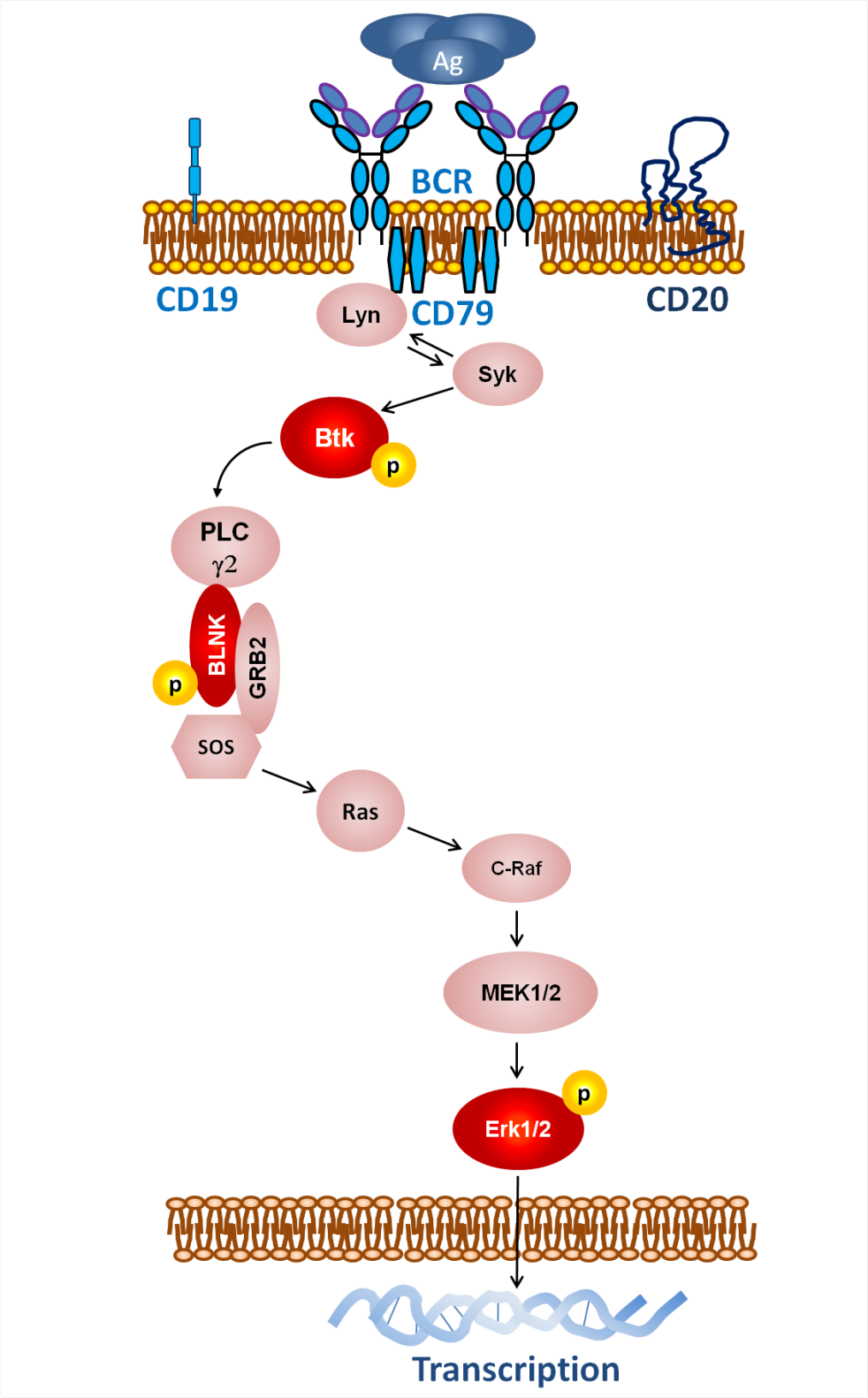
3.2.3 Inter and intra assay control

Inter and intra assay controls were used to maintain the experimental settings stable over time. PBMCs from a healthy volunteer sample (control samples) were divided in

aliquots and stored in liquid nitrogen. Measurement of inter assay control was performed as follows: An aliquot from the control sample was thawed and rested in RPMI-1640 (Sigma) supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 overnight. Next day, cells were stained with CD20-Pacific Blue (eBioscience), CD27-APC (eBioscience), CD24-PE (eBioscience), CD38-PECy7 (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 20 minutes at 37°C 5% CO_2. After the staining, PBMCs were divided in 6 5ml polystyrene tubes ($1.0x10^6$cells/tube) and activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) (Southern Biotech) for 0 and 10 minutes at 37°C. Cells were stained for ERK-p and samples were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA) and delta ERK-p was calculated for each subset. For intra assay control, an aliquot from the control sample was thawed, stained, activated and acquired every time an experiment with patients was performed. Delta ERK-p from patient samples was normalized with delta ERK-p from the control sample.

3.2.4 B cell functional assays in response to BCR activation measured in kidney transplant patient and healthy control's PBMCs

PBMCs from patients and healthy controls were thawed and rested in RPMI-1640 (Sigma) supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 overnight. Next day, cells were stained with CD20-Pacific Blue (eBioscience), CD27-APC (eBioscience), CD24-PE (eBioscience), CD38-PECy7 (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 20 minutes at 37°C 5% CO_2. After the staining, $3x10^6$ PBMCs were divided in 4 5ml polystyrene tubes and activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) (Southern Biotech) for 0, 10 and 30 minutes and with PMA (0.1uM, SIGMA) for 10 minutes at 37°C. Cells were fixed with BD Cytofix buffer (BD) for 10 minutes at 37°C, permeabilised with BD Phosflow Perm Buffer III (BD) for 30 minutes on ice and stained with anti-ERK1/2 (pT202/pY204)-AlexaFluor488 (BD) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours and data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA). Delta ERK-p was calculated in total B cell and B cell subsets from healthy control and patient samples, and their data was normalized using delta ERK-p obtained from the control sample.



Picture 6: **BCR signalling pathway showing proteins measured by phospho-flow in red**

3.2.5 Staining and isolation of membrane and cytosolic proteins

PBMCs of interest were thawed from liquid nitrogen the same day of protein preparation. MHC expression was enriched removing $CD2^+$ cells from PBMCs samples using anti-CD2 beads (Miltenyi). $20x10^6$ PBMCs were incubated with 100ul of anti-CD2 beads for 15 minutes at 4°C. Cells were washed, counted, and $CD2^-$ cells were stained with CFSE (5uM) for 20 minutes at 37°C. The reaction was stopped with 10ml of cold FBS for 5 minutes at 4°C and cells were washed two times with cold PBS. Stained cells were used to obtain proteins preparations. Membrane proteins from $CFSE^+$ cells were extracted with Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE). $5x10^6$ $CFSE^+$ cells (per eppendorf tube) were washed and resuspended in 150ul of Reagent A for 10 minutes at room temperature. Cells were washed and resuspended in 450ul of Reagent B/C for 30 minutes on ice vortexing every 5 minutes. Samples were centrifuged at 10,000xg for 3 minutes at 4°C and supernatants were removed and placed into new tubes for 10 minutes at 37°C to separate the membrane protein fraction. Samples were centrifuged again at 10,000xg for 2 minutes at room temperature to isolate the hydrophobic fraction containing membrane protein (bottom layer) from the hydrophilic fraction (top layer). The hydrophilic fraction was carefully removed and both fractions were collected in new tubes and stored at -80°C. The staining of membrane and hydrophilic proteins was checked by flow cytometry measuring the positive signal in the FITC channel. Membrane proteins ($\pm$100ul) were placed into Slide-A-Lyzer MINI Dialysis Devices, 10K MWCO (Thermo Scientific) for further dialysis. First, dialysis was performed during 3 hours at 4°C against 1000ml of 0.5% CHAPS 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (Biovision) dialysis buffer, then, dialysis buffer was changed and a second dialysis was performed overnight at 4°C. After dialysis, protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific). 25ul of sample replicates and standard were placed into a 96-well plate (Nunc™, Maxisorb, Thermo Scientific). After adding 200ul of BCA Working Reagent (WR), samples were mixed for 30 seconds and plate was covered and incubated at 37°C for 30 minutes. Plate was cooled at room temperature and optical density at 562nm was measured using Sunrise ELISA Reader (TECAN, UK). Samples tubes were labelled and stored at -80°C. Control samples were used to identify HLA class I and II in the membrane and hydrophilic fraction using western blot.

3.2.6 Identification of $CFSE^+$ membrane and cytosolic proteins by western blot

In order to perform western blot, equal protein concentration was adjusted in all samples. 20ug of proteins were mixed with 2X Laemmli Sample Buffer, and samples were boiled at 100°C for 5 minutes. Equal amount of proteins was loaded into the wells of the Mini Protean Precast Gels 4-15% gradient (BIO-RAD), including a molecular weight marker kaleidoscope pre-stained standard (BIO RAD). Electrophoresis was performed 2 hours at 100V in running buffer (Tris-Glycine/SDS: 25mM Tris-base (Fisher Scientific), 190mM Glycine (Fisher Scientific) and 0.1% SDS (SIGMA)). Proteins were transferred to a nitrocellulose membrane (BIO RAD for 2 hours at 120V using transfer buffer (48mM Tris (Fisher Scientific), 39mM glycine (Fisher Scientific), 20% methanol (SIGMA) and 0.04% SDS (SIGMA)). Transfer efficiency was checked using Ponceau Red staining (Thermo Scientific). Membrane was blocked for 1 hour at room temperature using blocking solution (3g of Skimmed Milk in 100ml PBS). Membrane was incubated with a primary Anti-MHC class I (ab52922) diluted 1/1000 (Abcam) antibody or Anti-MHC class II antibody (ab55152) diluted 1/1000 (Abcam) in blocking solution overnight at 4°C or for 2 hours at room temperature. Membrane was washed, three times with PBS 5% Tween20 (Sigma) for 5 minutes each, and incubated with a secondary anti-mouse IgG conjugated to HRP diluted 1/20000 (GE Healthcare, Life Sciences) antibody in blocking solution at room temperature for 1 hour. Membrane was washed, three times with PBS 5% Tween20 (Sigma) for 5 minutes each, covered with 2ml of Pierce ECL Western Blotting Substrate (Thermo Scientific) and wrapped in transparent film. Protein detection was performed exposing the membrane to a film (Amersham), and development was performed using Compact X4 Automatic X-ray Film Processor (Xograph).

3.2.7 Positive controls for protein capture

Two positive controls were used in the capture assay. The first control was a husband/wife pair sample in which the wife has been pregnant three times. In this case, the positive control corresponded to husband proteins recognised by wife B cells and the negative control corresponded to autologous wife proteins. $CFSE^+$ B cells were analysed by flow cytometry and ImageStream. The second control pair sample was a kidney transplant patient with known anti–HLA antibodies. In this case, the positive

control proteins were prepared from samples with MHC recognised by recipient antibodies and negative control was prepared from samples with MHC not recognised by recipient antibodies. In the second samples, $CFSE^+$ B cells and $CFSE^+$ monocytes were analysed by flow cytometry. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.2.8 Location of $CFSE^+$ donor-proteins

PBMCs from the first positive control were thawed and cultured overnight in complete media supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 before activation. The next day, 0.5ug/ml of $CFSE^+$ membrane and cytosolic proteins from the first positive controls (husband and wife) were cultured in the presence of $5x10^6$ PBMCs from the positive control sample (wife), 24 hours at 37°C. Next day, cells were harvested and $CFSE^+$ proteins were identified by flow cytometry and ImageStream. For flow cytometry, $1x10^6$ PBMCs were stained with anti-CD20-eFluor450 (eBioscience), anti-CD27-APC (eBioscience), anti-CD24-PE (eBioscience), anti-CD38-PECy7 (eBioscience) and Live/Dead yellow for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA). Regarding ImageStream, $9x10^6$ PBMCs were stained with anti-CD20-AlexaFluor780 (eBioscience), anti-HLA-DR-PE (eBioscience) and Live/Dead yellow for 30 minutes at 4°C. B cells were sorted as $Live/Dead^-CD20^+HLA\text{-}DR^+$ B cells using ARIA II (BD). Isolated B cell were analysed by ImageStream. Data was acquired on an $ImageStream^X$ system equipped with three lasers and running with Inspire software (both AMNIS Corp, Seattle, WA, USA). IDEAS 3.0 software (AMNIS) was used for analysis. Intensity of CD20 and HLA-DR was masked in the cell surface and localisation with $CFSE^+$ proteins was assessed using the internalisation feature between $CFSE^+$ proteins and $CD20^+HLA\text{-}DR^+$ intensity masked in the cell surface.

3.2.9 Identification of donor-proteins capture by recipient B cells from kidney transplant patients

PBMCs from recipients were thawed and cultured overnight in complete media supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 before activation. The next day, 0.1ug/ml of $CFSE^+$ membrane and cytosolic proteins from donor or recipient samples were cultured with $2.5\text{-}5x10^6$ PBMCs from the appropriate recipient samples

for 24 hours at 37°C. Next day, PBMCs were stained with anti-CD20-eFluor450 (eBioscience), anti-CD27-APC (eBioscience), anti-CD24-PE (eBioscience), anti-CD38-PECy7 (eBioscience) and Live/Dead yellow for 30 minutes at 4°C. Samples were analysed by flow cytometry to identify donor $CFSE^+$ proteins in recipient B cells. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.2.10 $CD4^+$ T cells antigen-specific activation mediated by B cells from kidney transplant patients and healthy controls

IFN-γ/IL-10 dual-ELISPOT (R&D) was used to measure antigen-specific activation on $CD4^+$ T cells elicited by autologous B cell subsets from kidney transplant patient. Cell preparation was performed as described below. PBMCs from recipients were thawed and cultured overnight in complete media supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 before activation. Next day, $CD4^+$ T cells were obtained by positive selection using anti-CD4 beads (Miltenyi). B cells were enriched by negative selection. $CD2^+$, $CD3^+$ and $CD14^+$ cells were removed from PBMCs samples using anti-CD2, anti-CD3 and anti-CD14 beads (Miltenyi). Enriched B cell samples from recipients were stained with anti-CD20-eFluor450 (eBioscience), anti-CD27-APC (eBioscience), anti-CD24-PerCP-Cy5.5 (BD), anti-CD38-PECy7 (eBioscience) and Live/Dead yellow (Invitrogen) for 30 minutes at 4°C. Cells were washed and B cell subsets from recipients were sorted using ARIA II (BD). Memory B cells were $CD20^+CD27^+$ (purity >95%), Naïve B cells were $CD20^+CD27^-CD24^+CD38^+$ (purity >95%) and Transitional B cells were $CD20^+CD27^-CD24^{hi}CD38^{hi}$ (purity >95%).

IFN-γ/IL-10 dual-ELISPOT plate (R&D) was blocked using 200ul of complete media for 30 minutes at room temperature. Plates were washed and 100ul containing $1x10^5$ recipient $CD4^+$ T cells in complete media supplemented with IL-2 (100ng/ml, R&D) were added per well. $1.0x10^5$ $CD4^+$ T cells were cultured alone, with $0.5x10^5$ autologous Memory, Naïve or Transitional B cells with and without 0.1ug/ml of $CFSE^+$ membrane and cytosolic proteins obtained from donor samples during 72 hours at 37°C 5% CO_2. After 3 days, cells were collected and CD25 and CD86 activation markers were measured in $CD4^+$ T cell and $CD20^+$ B cells, respectively, by surface staining using anti-CD25-PE (eBioscience) and anti-CD86-FITC (eBioscience) antobodies. Cells

were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

IFN-γ/IL-10 dual-ELISPOT plate was washed 3 times with 300ul/well of wash buffer, 100ul/well of diluted detection antibody mixture was added, and the plate was incubated overnight at 4°C. Next day, the plate was washed and 100ul/well of diluted Streptavidin-AP was added for 2 hours at room temperature. Plate was washed again, and 100ul/well of BCIP/NBT chromogen was added for 1 hour at room temperature protected from light. BCIP/NBT chromogen was decanted and rinsed with deionised water. Plate was then inverted to remove excess water. 100ul/well of AEC chromogen solution was added for 20 minutes at room temperature protected from light. AEC chromogen solution was decanted and rinsed with deionised water. Plate was inverted to remove excess water and the flexible plastic under-drain was removed. After draying the plate at room temperature for 90 minutes, IFN-γ and IL-10 spots were counted using The AID iSpot reader system (AID, Oxford Biosystems Cadama).

3.2.11 HLA antibodies detection

Aliquots of frozen serum samples from kidney transplant patient were thawed just before the assay. Aggregates were removed from the serum by centrifugation (8,000-10,000G for 10 minutes) or filtration (0.2mm) prior to testing. LABScreen (One Lambda, Inc) beads were mix by gently vortexing or pipetting up and down several times prior to use. After mixing, 5ul of beads were incubated with 20ul of serum sample in a 96-well plate for 30 minutes in the dark at 25º C with gentle shaking. After incubation, plate was washed two times with 1X wash buffer. The plate was covered with tray seal (OLI Cat. #SSPSEA300), agitated and centrifuged 1,300G for 5 minutes to remove excess of wash buffer. 1X of PE-conjugated anti-human IgG (OLI Cat # LS-AB2) was incubated in the dark for 30 minutes at 25º C. The plate was washed to remove the excess of PE-IgG and 80ul of PBS was added per well. The sample was ready for data acquisition and analysis. Data was acquired in a Luminex LX100 (Luminex Corp., Austin, TX) and analysis was performed using HLA Fusion^{TM} software (OneLambda).

3.3 The effect of B cell subsets as $CD4^+$ T cells-activating cells.

3.3.1 Cell culture media

All cell cultures were grown using RPMI-1640 (Sigma) supplemented with 2mM L-Glutamine (Gibco, Invitrogen), 100U/mL penicillin, 100mg/mL streptomycin (Gibco, Invitrogen) and 10% of Foetal Calf Serum (FCS) (SeraQ, Sussex, UK).

3.3.2 Isolation of $CD4^+$ T cell and $CD20^+$ B cell from peripheral blood (cones)

Peripheral blood (cones) from healthy volunteers was used to obtain $CD4^+$ T cells and $CD20^+$ B cells. Samples were diluted 1:2 with PBS 2% Bovine Serum Albumin (BSA) and 50ul/ml of RosettSep Human B cell enrichment cocktail and RosettSep Human $CD4^+$ T cell enrichment cocktail (STEM CELL) was added to the corresponding sample for 20 minutes at room temperature. Samples were diluted again 1:4 with PBS 2% BSA and $CD4^+$ T cells and $CD20^+$ B cells were obtained directly from the mononuclear fraction obtained after Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells and $CD20^+$ B cells were washed two times, counted and resuspended in PBS.

3.3.3 Preparation of $CD4^+$ T cell

$CD4^+$ T cells were stained with 1uM CellTrace Violet Cell Proliferation Kit (Invitrogen) for 20 minutes at 37°C protected from light. Unbound dye was quenched adding cold FCS for 5 minutes at 4°C. $CD4^+$ T cells were washed two times, resuspended in complete media supplemented with IL-2 (100ng/ml, R&D) and placed in anti-CD3 (1ug/ml, SIGMA) pre-coated plates. $1x10^6$ of $CD4^+$ T cells/well and $0.5x10^6$ of $CD4^+$ T cells/well were cultured 6 hours in anti-CD3 pre-coated plates before the addition of the B cell subsets.

3.3.4 $CD20^+$ B cell sorting

B cells were counted and $60x10^6$ cells were resuspended in 200ul of PBS. B cells were stained with anti-CD20-Pacific Blue (eBioscience), anti-CD27-APC (eBioscience), anti-CD24-PerCP-Cy5.5 (BD), anti-CD38-PECy7 (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 30 minutes 4°C. B cell subsets were sorted with ARIA II (BD). Memory B cells were $CD20^+CD27^+$ (purity >95%), Naïve B cells were $CD20^+CD27^-CD24^+CD38^+$ (purity >95%) and Transitional B cells were $CD20^+CD27^-CD24^{hi}CD38^{hi}$ (purity >95%) (Table 4).

3.3.5 $CD4^+$ T cell - B cell subsets co-cultures

Large Scale experiment: $1x10^6$ $CD4^+$ T cells/well were cultured with and without $0.5x10^6$ sorted B cell, Memory, Naïve or Transitional B cells in 48 well-plates for 5 days at 37°C 5% CO_2. $1x10^6$ $CD4^+$ T cells/well were cultured with $0.5x10^6$ B cell subsets in the presence or absence of 0.1ng/ml anti-IL-10R (R&D).

Time Course experiments: $0.5x10^6$ $CD4^+$ T cells/well were cultured with and without $0.5x10^6$ sorted B cell, Memory, Naïve or Transitional B cells in 96 well-plates for 1, 3 and 5 days at 37°C 5% CO_2.

After culture, $CD4^+$ T cells and B cell subsets were collected and tested for $CD4^+$ T cell proliferation, $CD4^+$ T cell and $CD20^+$ B cell activation, $CD4^+$ T cell and $CD20^+$ B cell cytokine production and $CD20^+$ B cell viability.

3.3.5.1 Flow cytometry: Proliferation

$CD4^+$ T cells were previously stained with CellTrace Violet Cell Proliferation Kit (Invitrogen). Cells from T cell - B cell co-cultures were stained with anti-CD20-AlexaFluor780 (eBioscience) and anti-CD3-PerCP-Cy5.5 and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.3.5.2 Flow cytometry: Activation markers

Cells from T cell -B cell co-cultures were stained with anti-CD20-AlexaFluor780 (eBioscience) and anti-CD3-PerCP-Cy5.5 (BD), anti-CD25-APC (eBioscience), anti-CD69-FITC (eBioscience), anti-CD86-PE (eBioscience), anti-CD40L-PE (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.3.5.3 Flow cytometry: Cytokine Production

Intracellular staining and ELISA were performed to measure IL-10, IFN-γ and TNF-α. Cells were activated with PMA (50ng/mL), ionomicyn (1ug/mL), GolgiStop (BD) and Brefeldin A (BD) for 5 hours in new culture media at 37°C 5% CO_2. Cells were fixed and permeabilised with $FoxP_3$ Fix and Perm Kit (BD) and stained with IL-10-PE (BD),

IFN-γ-APC (eBioscience) and TNF-α-PECy7 (eBioscience) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.3.5.4 Flow cytometry: B cell viability

Live/Dead fixable yellow dye up-take and expression of BAFFr were used to assess cell death and apoptosis in the B cell subsets. Cells from T cell - B cell co-cultures were stained with anti-CD20-AlexaFluor780 (eBioscience) and anti-CD3-PerCP-Cy5.5, anti-BAFFr-FITC (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) for 30 minutes at 4°C. Cells were acquired on an LSRFortessa (BD) within 24 hours. Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

3.4 Genes differentially expressed in B cells from kidney transplant patients and healthy controls.

3.4.1 B cells isolation from kidney transplant patients and healthy controls

PBMCs samples were thawed from liquid nitrogen the same day of the staining. $CD20^+$ B cells were enriched by negative selection with anti-CD3, anti-CD4 and anti-CD14 beads (Miltenyi). Enriched B cell samples from recipients and healthy controls were stained with anti-CD20-eFluor450 (eBioscience) and Live/Dead yellow (Invitrogen) for 30 minutes at 4°C. Cells were washed and live B cells were sorted using ARIA II (BD). $CD20^+$ B cells purity was >95%. B cells were then washed with PBS two times and resuspended in 1ml of Trizol (BioLine). Vials containing kidney transplant recipients and healthy controls B cell samples in Trizol were shipped to Miltenyi Biotec on dry ice in two shipments.

3.4.2 Quality control (QC) of total RNA

RNA was isolated using standard RNA extraction protocols. RNA samples were quality-checked *via* the Agilent 2100 Bioanalyzer platform (Agilent Technologies). The results of the Bioanalyzer run were visualised in a gel image and an electropherogram using the Agilent 2100 Bioanalyzer expert software (data not shown). In addition to this visual control, the software generated an RNA Integrity Number (RIN) to check integrity and overall quality of total RNA samples. The RIN value was calculated by a proprietary algorithm that takes several QC parameters into account, for example, 28S RNA/18S RNA peak area ratios and unexpected peaks in the 5S RNA region. A RIN number of 10 indicated high RNA quality, and a RIN number of 1 indicates low RNA quality. According to published data and Miltenyi Biotec experience, RNA with a RIN number >6 is of sufficient quality for gene expression profiling experiments.

3.4.3 Linear T7-based amplification of RNA

For the linear T7-based amplification step, 20-50ng (depending on the available amount) of each total RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labelled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the

dye-incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies).

3.4.4 Hybridisation of Agilent Whole Genome Oligo Microarrays

The hybridisation procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 600ng Cy3-labeled fragmented cRNA in hybridisation buffer was hybridised overnight (17 hours, 65°C) to Agilent Whole Human Genome Oligo Microarrays 8x60K using Agilent's recommended hybridisation chamber and oven. Finally, the microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 minute at room temperature followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37°C) for 1 minute. The last washing step was performed with acetonitrile.

3.4.5 Scanning results

Fluorescence signals of the hybridised Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies).

3.4.6 Image and data analysis

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities (including background subtraction), rejects outliers and calculates statistical confidences. Data analysis was performed by the statistician from the GAMBIT study.

3.5 Statistical analysis

Statistical analysis tests were used according to the experimental design of each experiment, and further multiple comparison tests were performed to detect differences between variables.

Non-parametric Kruskal-Wallis test with a Dunn's multiple comparisons was used to analyse phenotypic studies and ERK phosphorylation in healthy controls and kidney transplant patient samples. This test was used because these experiments had one nominal variable (patients groups) and one measurement variable (percentages of populations), and the measurement variable was not normally distributed; * $p<0.05$ was considered significant.

Wilcoxon matched-pairs signed rank test was used to measure differences in cytokine production from "Loss Tolerance" kidney transplant patient between non-activated and activated samples. This test was use for the same reason than the Kruskal-Wallis test, but in this case, only two groups were compared; * $p<0.05$ was considered significant.

Non-parametric Friedman test with a Dunn's multiple comparisons was used to analyse CD20 and CD19 MFI on B cells subsets from healthy controls and kidney transplant patient samples and IL-10 receptor expression on the B cell subsets. This statistical analysis was used to test continuous data when the variable was not normally distributed; * $p<0.05$ was considered significant.

Two-way RM ANOVA test with Holm-Sidak, Sidak or Tukey's multiple comparisons was used to evaluate differences between non-activated and activated samples from healthy controls and kidney transplant patient and differences between B cell subsets. This test was used because the same variable was investigated over two or more time points in the same group; * $p<0.05$ was considered significant.

Data collection was carried out using Microsoft Excel (Microsoft) and tabulated data was transferred to Prism 6 (GraphPad Software, Inc). Data tables, statistical analysis and graphs were performed using Prism 6 (GraphPad Software, Inc).

4 Phenotypical and functional characterisation of B cell subsets in kidney transplant patients

4.1 Introduction.

The role of B cells in kidney transplantation has been traditionally associated with the recognition of graft proteins by alloantibodies produced by B cell-differentiated Plasma cells (Mohan *et al*. 2012). These alloantibodies can adversely affect graft survival before and after transplantation. Before transplantation, the presence of pre-existing alloantibodies can induce hyperacute rejection and accelerated acute rejection (Loupy *et al*. 2012); after transplantation, *de novo* alloantibodies can also trigger a detrimental immune response in chronic graft rejection, *via* donor-antigen recognition (Hidalgo *et al*. 2009). In addition, alloantibodies can compromise graft survival in early acute and chronic rejection, through the activation of the classical complement pathway (Csencsits *et al*. 2008). In summary, alloantibodies are recognised as negative indicators of graft survival, and their presence is predominantly associated with rejection.

Alongside alloantibody production, B cells can also affect kidney transplantation success by acting as professional APCs. The role of these cells as APCs begins when protein from donor cells are first recognised by the BCR expressed on the cell surface of Naïve B cells (Monroe 2006). Once the protein is bound and the BCR–protein complex is internalised, the cell machinery processes donor-proteins, which are then presented as peptides in the context of MHC class II (LeBien *et al*. 2008). Donor-antigens are recognised by alloantigen-specific $CD4^+$ T cells from the recipient *via* MHC class II–TCR interaction. This interaction, known as indirect recognition, occurs only when B and T cells share the same antigen-specificity. After MHC class II-TCR ligation, several activation proteins are up-regulated in both the B cell and T cell such as CD25, CD86, CD69 and so on (Clark *et al*. 1994). Interactions between these proteins induce activation of both cells. Finally, activated $CD4^+$ T cells help Naïve B cells to become Memory or Plasma cells. Memory B cells are the more efficient APCs, because they exhibit high antigen specificity, compared to Naïve B cells, and Plasma cells will produce donor-specific antibodies; both cells can participate in a rejecting immune response (Kimball *et al*. 2011).

As a consequence of their detrimental role in kidney transplantation, B cells have been targeted in depletion therapies, with the aim to improve kidney function and graft survival. Removal of B cells increases transplantation success by blocking the adverse effects produced by alloantibodies. While this has been demonstrated in some studies reporting an improvement in graft survival (Becker *et al*. 2004; Alausa *et al*. 2005; Bagnasco *et al*. 2007), others have reported conflicting results (Clatworthy *et al*. 2009). The effectiveness of CD20-depletion therapy therefore is still a controversial field and further study is required to clarify the benefits of targeting B cells as their role in transplantation tolerance remains unclear.

Transplantation tolerance has been defined as long-term acceptance of transplanted organs without requirement for indefinite immunosuppression (Nickerson *et al*. 1994; Dong *et al*. 1999). Although the role of B cells in transplantation tolerance is unclear, several groups have now identified a link between B cells and kidney tolerant patients. These patients have been defined as transplant recipients that maintain a stable graft function without immunosuppressant therapy. Three independents studies (Newell *et al*. 2010; Pallier *et al*. 2010; Sagoo *et al*. 2010) demonstrated an expansion of B cells, and a high expression of B cell-related genes in tolerant recipients. These findings revealed that B cells cannot only be considered as a detrimental player in graft survival as previously described, they can also have a regulatory role in transplantation.

Despite the fact that antibody production and antigen presentation are the main functions performed by B cells, a specific human B cell subset with anti-inflammatory properties has been reported and defined as Transitional or Regulatory B cells. These cells are the first population to emerge from the bone marrow, and can be found in peripheral circulation exhibiting a specific phenotype characterised by high expression of CD24 and CD38 molecules (Sims *et al*. 2005). Their main anti-inflammatory property is IL-10 production that is mediated through CD40-CD40L interaction (Blair *et al*. 2010).

The identification of a B cell subset with regulatory properties is particularly relevant in humans and opens a new interest in the role of Transitional or Regulatory B cells in kidney transplantation tolerance. So far, our research group has reported an expansion of total B cells in peripheral blood, the absence of donor-specific antibodies in serum, and a differential expression of B cell-related genes in tolerant patients (Sagoo

et al. 2010). The first aim of this thesis was to quantify the B cell subsets, particularly Transitional B cells, in the peripheral blood samples from kidney transplant recipients and healthy controls. A clear expansion of specific B cell subsets in tolerant patients was not addressed at the beginning of this thesis since two contradictory sets of data were published. The first, from Brouard's group, showed that Memory B cells were the main players in transplant tolerant patients (Pallier *et al*. 2010), while Newell *et al*. reported that Naïve and Transitional B cells are the main regulatory cells (Newell *et al*. 2010). The focus of this thesis was directed towards the characterisation of B cells using markers previously reported (Sims *et al*. 2005; Marie-Cardine *et al*. 2008; Palanichamy *et al*. 2009), specifically towards identified Memory, Naïve and Transitional B cells.

In the same papers mentioned above, *MS4A1,* the gene that encodes the CD20 molecule, was the only common gene up-regulated in tolerant patients. *MS4A1* was the fifth gene differentially expressed in tolerant recipients in the IOT study (Sagoo *et al*. 2010). It was up-regulated in tolerant patients from Brouard's study (Brouard *et al*. 2007; Pallier *et al*. 2010), and was also up-regulated in tolerant patients from the ITN study (Newell *et al*. 2010). Moreover, in the latter study, the CD20 transcript was the only gene significantly expressed at high levels in urine sediments from tolerant patients, compared to patients with stable function and healthy controls (Newell *et al*. 2010). Interestingly, Transitional B cells express a significantly higher CD20 MFI, compared to Naïve and Memory B cells (Cuss *et al*. 2006). Hence, the CD20 findings in tolerant recipients can be explained by an increase of Transitional B cells in tolerant recipients or by a potential, and as yet undiscovered, role of CD20 in transplantation tolerance. To answer this question, part of the phenotypic study was focused on the CD20 expression in B cells from healthy controls and transplant patients.

The main difference between the groups of patients was the absence of immunosuppressants in tolerant recipients. To investigate whether the presence of immunosuppressive drugs in circulation could have affected the B cell signature, apoptosis induction and cell death was studied in samples from healthy volunteers cultured with the main immunosuppressive drugs used in kidney transplantation: tacrolimus, MMF and steroids.

Finally, B cell activation and anti-inflammatory responses, ascertained by IL-10 production, were evaluated in kidney transplant patients and healthy controls. CpG and

CD40L are the main stimuli to induce IL-10 production in human B cells. Whereas CpG activation induced IL-10 secretion from total B cells (Liang *et al*. 2010), CD40 activation targeted Transitional (Blair *et al*. 2010) and Naïve cells (Duddy *et al*. 2004; Iwata *et al*. 2011).

In summary, B cells in kidney transplantation are not only predecessors of antibody-producing cells and professional APCs; their contribution as cytokine-producing cells is also relevant when describing their role in transplantation tolerance. Since IL-10 is the main cytokine in peripheral tolerance and Transitional B cells are the main IL-10-producing B cells after CD40 activation (Blair *et al*. 2010), the first hypothesis to cover was:

- Transitional B cells are expanded in peripheral blood of tolerant kidney transplant recipients.

To address this hypothesis, the percentage of the B cell subsets was measured in kidney transplant recipients and healthy controls. Once B cell subset distribution was established, the second hypothesis was considered:

- Transitional B cells from tolerant patients modulate an anti-inflammatory response and therefore participate in the state of tolerance.

To answer the second hypothesis, expression of B cell activation markers and cytokine production, after CD40L and CpG interaction, was measured in kidney transplant patients and healthy controls to establish if tolerant recipients were exerting an IL-10-mediated regulatory response.

4.2 Phenotypic characterisation of B cell subsets in kidney transplant patients.

4.2.1 B cell phenotype and the identification of B cell subsets

In order to identify the B cell profile in the different groups of patients, an 8-colour flow-cytometry panel was developed to analyse B cell subsets within PBMCs from frozen samples. Age and gender-matched healthy controls were used as a reference for normal B cell subset distribution.

The flow panel was developed using antibodies for selected B cell surface markers and BCR components based on several publications (Sims *et al*. 2005; Marie-Cardine *et al*. 2008; Lee *et al*. 2009; Palanichamy *et al*. 2009). The purpose of the staining was to identify Memory, Naïve and Transitional B cells with high precision.

Anti-CD20 and anti-CD19 antibodies were used to perform a double staining to identify B cells within PBMCs (Fig1 A). From the $CD20^{+}CD19^{+}$ population, a specific antibody for the CD27 molecule was used to separate Memory B cells ($CD27^{+}$) from $CD27^{-}$ B cells (Fig1 B) (Agematsu *et al*. 1997; Agematsu *et al*. 1998). From the $CD27^{-}$ fraction, IgM/IgD double positive cells were selected (Fig1 C) because Transitional B cells have been previously described as cells expressing high levels of IgD and IgM (Sims *et al*. 2005). Finally, specific antibodies for CD24 and CD38, two markers highly expressed in Transitional B cells, were used to identify three $CD27^{-}$ B cell subsets (Fig1 D). Transitional B cells were identified through their characteristic elongated triangle distribution due to their linear co-expression of CD38 and CD24 (Cuss *et al*. 2006; Lee *et al*. 2009; Blair *et al*. 2010). According to the maturation process, Naïve B cell, identified as $CD24^{+}CD38^{+}$, lose the high expression of CD24 and CD38, but maintain a positive signal for both markers. Finally, $CD24^{+}CD38^{-}$ Mature B cells completely lose CD38 but maintain CD24 expression.

Without forgetting the role as antibody-producing cells, the levels of pre-Plasma cells in circulation were measured. To achieve this, $CD20^{-}CD19^{+}CD27^{+}CD38^{hi}$ pre-Plasma cells (Fig1 F) were selected from the $CD20^{-}CD19^{+}$ population (Fig1 E).

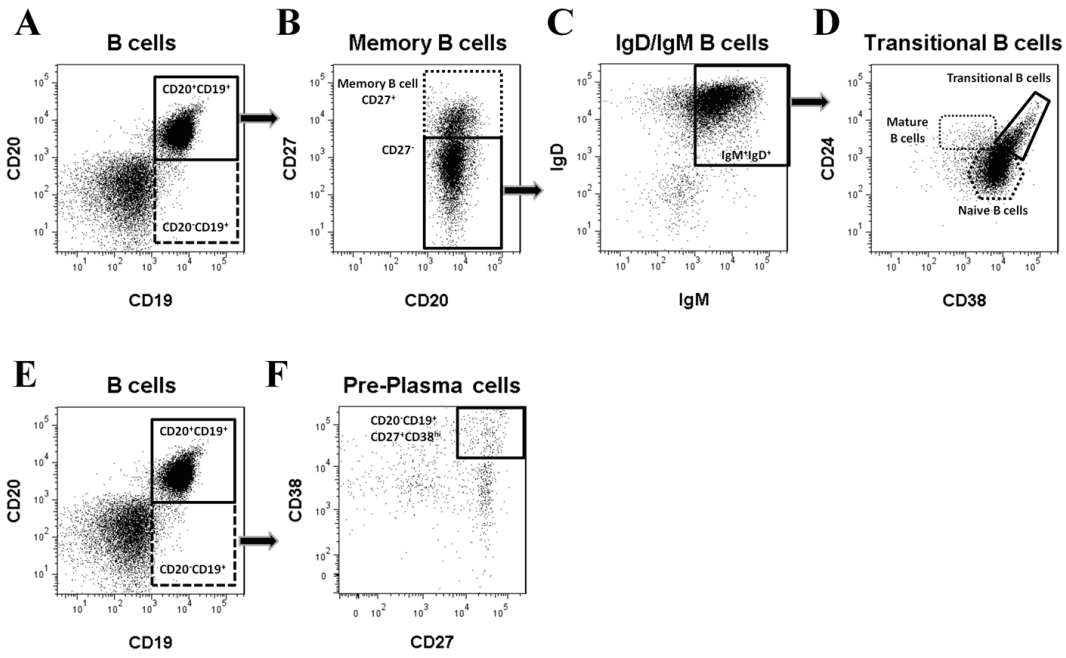


Figure 1: B cell subset identification.

Samples were obtained from frozen PBMCs separated from the mononuclear fraction of whole blood after gradient centrifugation. Samples were thawed on the same day of the experiment and stained with the 8-colour flow-panel. Fortessa settings were maintained stable during three years of study using Cytometer Setup and Tracking Beads (CS&T), application settings and an internal assay control, which was prepared and acquired at the same time than patient samples. B cells were identified as $CD20^{+}CD19^{+}$ cells by a double staining for CD20 and CD19 (A). From the $CD20^{+}CD19^{+}$ population, Memory B cells were identified as $CD27^{+}$ B cells (B), and from the $CD27^{-}$ population, $IgD^{+}IgM^{+}$ B cells were then selected (C). From the $CD27^{-}IgD^{+}IgM^{+}$, Transitional B cells were identified as $CD24^{hi}CD38^{hi}$, Naïve B cells were identified as $CD24^{+}CD38^{+}$ and Mature B cells were identified as $CD24^{+}CD38^{-}$ (D). From the $CD20^{-}CD19^{+}$ population (E) pre-Plasma cells were identified as $CD27^{+}CD38^{hi}$.

4.2.2 Distribution of B cell subsets in peripheral blood, spleen and lymph nodes

Transitional B cells are the first population that emerge from the bone marrow and enters into circulation. Once in the periphery, Transitional B cells have the potential to become Naïve B cells and continue their maturation process, hence, peripheral blood should be the main source for locating these cells. However, it is not clear if this population can migrate into immune organs, like spleen or lymph nodes, to play a role as an independent regulatory population.

In order to find the main location of Transitional B cells and the remaining B cell subsets, the B cell panel was performed in three different tissues: peripheral blood, spleen and lymph nodes (Fig 2). In Figure 2A, within the lymphocyte population, the percentage of B cell was 10-15% in peripheral blood, 50-60% in spleen and 40-50% in lymph nodes (Fig2 A). In terms of the different subsets, only Memory B cells were significantly increased in spleen (***$p<0.0001$) compared to peripheral blood. No significant difference was observed in the rest of the subsets between the tissues (Fig2 B).

Interestingly, even when the percentage of total B cells was three times as high in the spleen and lymph nodes when compared to circulation, the percentage of Transitional B cells was always higher in peripheral blood. Percentages of Transitional B cells in circulation were around 10-12%, whereas in spleen and lymph node they did not exceed 2% (Fig2 B).

Unlike Transitional B cells from spleen and lymph nodes, cells obtained from peripheral blood exhibited a clear and distinctive phenotype (described in Fig1 D), characterised by high expression of CD24 and CD38. The shape of this phenotype was absent in samples from spleen and lymph nodes (Fig2 C).

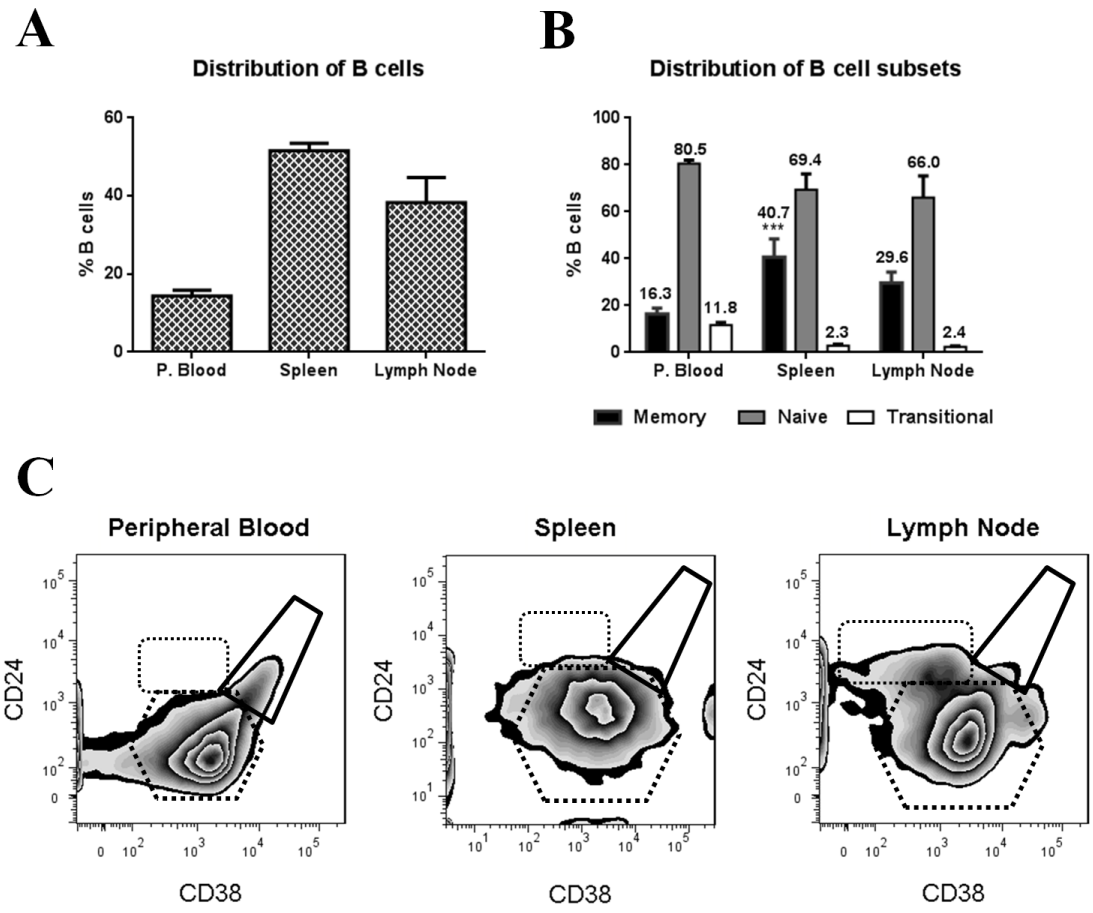


Figure 2: B cell subset distribution in peripheral blood, spleen and lymph nodes.

Ten frozen cells from peripheral blood samples of healthy volunteers, and six spleen and two lymph nodes samples from cadaveric donors were thawed and stained (as described in Fig 1) to identify B lymphocytes (A) and their subsets (B). Two-way RM ANOVA test with a Tukey's multiple comparisons test was used; * $p<0.05$ was considered significant. Examples from Transitional B cells in peripheral blood, spleen and lymph node are shown in counter plots (C).

4.2.3 Tolerant recipients exhibited higher percentages of Transitional B cells compared to non-tolerant recipients

Having established how to identify B cell subsets in samples from healthy volunteers, the distribution of the same subsets was then analysed in peripheral blood samples from kidney transplant patients (tolerant, stable, monotherapy, chronic rejector) and age-gender matched healthy controls.

The first analysis was performed to evaluate total B cells and the distribution of B cell populations in the different patient groups. The percentages of total B cells from healthy controls (*p=0.0140) and tolerant patients (**p=0.0017) were both significantly increased compared to B cells from stable patients (Fig3 A). Despite previous publications reporting a decreased number of B cells in chronic rejector, compared to tolerant and healthy controls, no differences in percentages were observed between these groups in this study. The difference in the results could be explained by the fact that the previous studies compared B cell numbers, whereas this study compared B cell percentages, between patients. No differences in percentages of total B cells were observed between healthy controls and tolerant patients ($p>0.05$); this result suggests that B cells are not "expanded" in tolerant recipients, compared to healthy controls.

B cell subsets (Memory, Naïve, Transitional, Mature and pre-Plasma B cells) were then analysed. The percentage of $CD27^+$ Memory B cells in the peripheral blood was around 20%, and no significant differences were observed between groups (Fig3 B). Naïve or $CD27^-CD24^+CD38^+$ B cells were the largest B cell population in the peripheral blood, but again, no difference between the percentages of total Naïve B cells amongst the patient groups was found (Fig3 C).

Although Transitional B cells were the smallest population within the B cell subsets, significant differences were found between patient groups. Transitional B cells were significantly expanded in tolerant patients, compared to stable patients (**p=0.0037) and chronic rejector (****p<0.0001) (Fig3 D).

Transitional B cells were also expanded in healthy volunteers in comparison to stable patients (**p=0.0032) or chronic rejector (****p<0.0001), but again, no difference in percentages of Transitional B cells between healthy volunteers and tolerant patients were observed ($p>0.05$).

Mature B cells, an interesting population usually absent in samples from healthy volunteers, was increased in peripheral blood from stable patients in comparison to healthy controls (*p=0.0341) and tolerant patients (*p=0.0305) (Fig3 E). Pre-Plasma cells were also increased in stable patients when compared to chronic rejector (*p=0.0468) (Fig3 F).

In summary, similar percentages were observed between healthy controls and tolerant patients. In fact, no significant differences were found in any B cell subset between these two groups. These results demonstrate that the percentages of B cells and their subsets were not greatly expanded in tolerant patients. However, percentages of Transitional B cells were increased in tolerant recipients, compared to patients with triple immunosuppressive therapy and chronic rejector. These results naturally led the direction of the investigation towards the potential effects of immunosuppressive drugs on the distribution of B cell subsets.

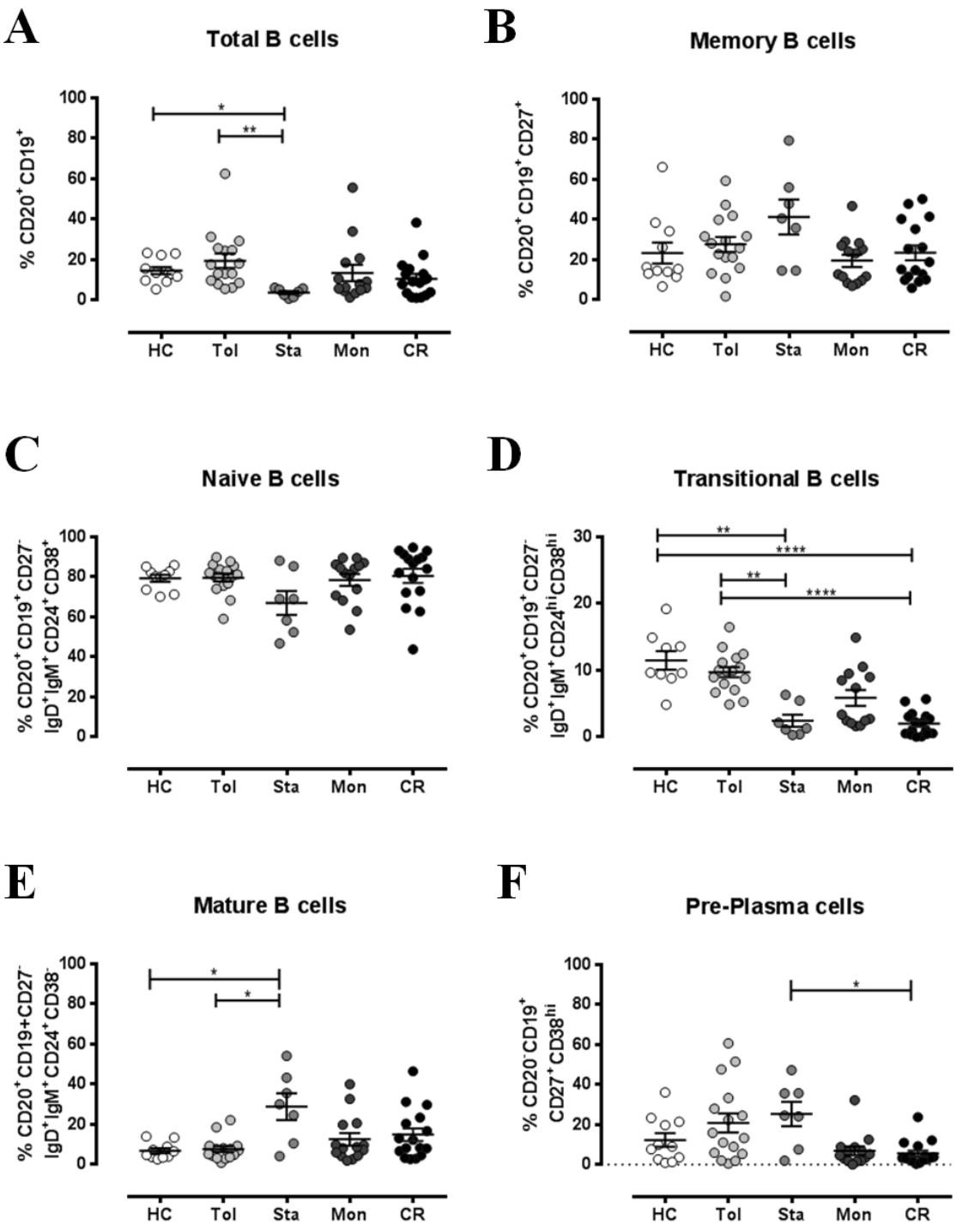


Figure 3: B cells subsets distribution in kidney transplant patients.

Percentages of total B cells (A), Memory (B), Naïve (C), Transitional (D), Mature (E) and pre-Plasma cells (F) were measured in PBMCs from healthy control (HC) tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR) (described in Table 3) by surface staining. Kruskal-Wallis test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.2.4 Effects of drugs on B cell viability

Having shown that the percentage of Transitional B cell was higher in tolerant patients and healthy controls when compared to the other groups under immunosuppressive therapy, a possible detrimental influence of these drugs in B cell viability or apoptosis was studied. Percentages of apoptotic B cells in healthy controls and kidney transplant patients were evaluated to identify any failure in the B cell survival. The aim of this experiment was to dismiss the possibility that immunosuppressive drugs were producing a specific decrease in the Transitional population in non-tolerant recipients.

An assay was developed to measure dead and apoptotic cells using a Live/Dead dye, AnnexinV and BAFFr. Firstly, live cells were identified as Live/Dead$^-$, whereas dead cells were identified as Live/Dead$^+$ (Fig4 A). Then, from the live population, apoptotic cells were identified as AnnexinV$^+$ BAFFr$^-$ (Fig4 B). It was demonstrated that down-regulation of BAFFr was a specific indicator of B cell apoptosis because apoptotic B cells, or AnnexinV$^+$ cells, were also BAFFr$^-$ (Fig4 B). Once the staining was set up, expression of BAFFr was evaluated in samples from healthy controls and kidney transplant patients. Results revealed that B cells from healthy controls exhibited a higher expression of BAFFr, compared to B cells from stable patients (*p=0.0280) and chronic rejector (*p=0.0404) (Fig4 C). Therefore, it can be suggested that apoptotic B cells were increased in these two patient groups.

The effect of tacrolimus, MMF and steroids in cell viability and apoptosis induction was measured using the same parameters mentioned above. The concentration range used started from drug concentrations found in patients' circulation, 0-10ng/ml for tacrolimus, 0-10ug/ml for MMF and 0-50ng/ml for steroids, to exceeding the therapeutic concentrations of patients, >10ng/ml for tacrolimus, >10ug/ml for MMF and >50ng/ml for steroids. Cell death was evaluated in total B cells (Fig5 A), Memory (Fig5 C), Naïve (Fig5 E) and Transitional B cells (Fig5 G). At the same time, apoptosis was also measured in total B cells (Fig5 B), Memory (Fig5 D), Naïve (Fig5 F) and Transitional B cells (Fig5 H).

The results obtained demonstrated that high concentration of steroids induced cell death mainly in Memory B cells (Fig5 C), whereas apoptosis occurred predominantly in Naïve (Fig5 F) and Transitional B cells (Fig5 H). No differences in B

cell viability were observed with tacrolimus or MMF.

Due to the effects observed with steroids, cell death was evaluated in the B cell subsets from samples treated with and without steroids (Fig6 A). Results showed that at high-drug concentrations, Memory B cells were the main population affected by this drug. Apoptosis was then measured in the B cell subsets (Fig6 B & C), and at 500ng/ml of steroids, Naïve and Transitional, but not Memory B cells, increased significantly their apoptotic cell percentages.

In conclusion, tacrolimus, MMF and steroids had no effect on cell viability or apoptosis induction at physiological concentration. On the other hand, when steroids were used at non-physiological concentrations, cell death or apoptosis was induced in Memory, Naïve and Transitional B cells.

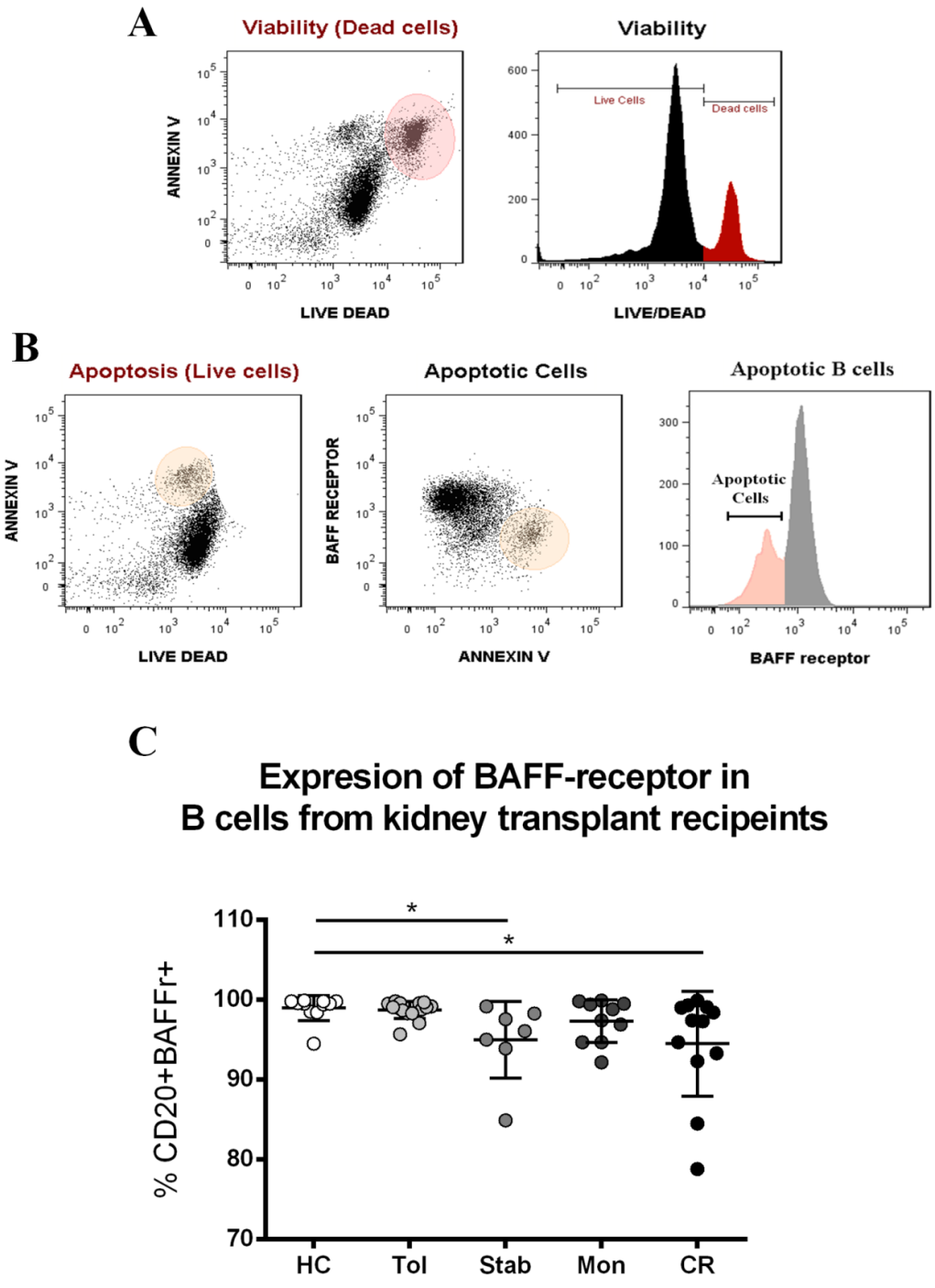


Figure 4: Measurement of B cell viability and apoptosis in kidney transplant patients.

Samples from healthy volunteers were used to identify dead B cells as Live/Dead^{+} , using flow cytometry (dot-plot and histogram) (A). From the Live/Dead^{-} population observed in Figure A, two dot-plots and one histogram were used to identify apoptotic B cells. The first dot-plot identified apoptotic B cells as AnnexinV^{+}Live/Dead^{-} . These AnnexinV^{+} Live/Dead^{-} cells were then plotted in an AnnexinV/BAFFr dot-plot, and images showed that apoptotic AnnexinV^{+} B cells were also BAFFr^{-} cells. Finally, a histogram showed AnnexinV^{+}BAFFr^{-} or apoptotic cells in orange (B). Expression of BAFFr

was measured in healthy control (HC) tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR). Kruskal-Wallis test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant.

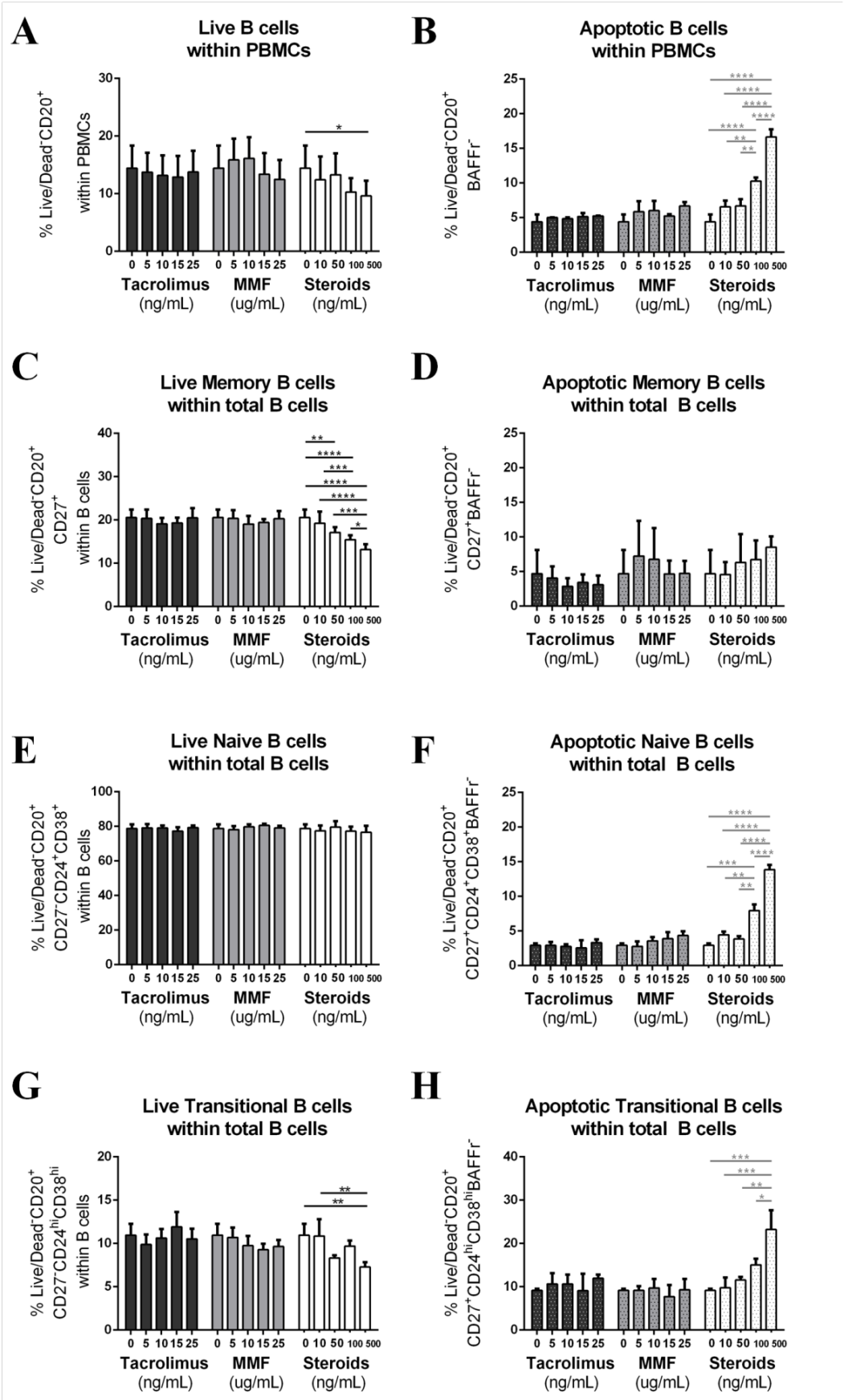
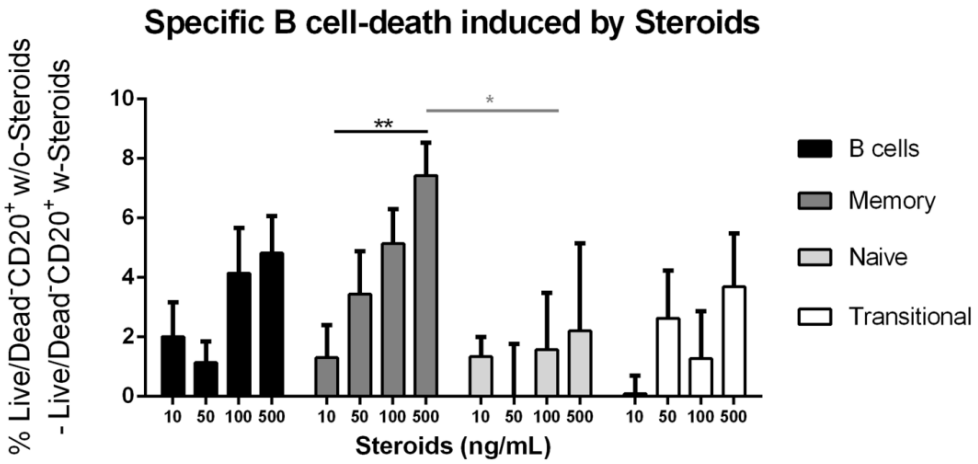

A
Live B cells within PBMCs
% Live/Dead-CD20+ within PBMCs
B
Apoptotic B cells within PBMCs
% Live/Dead-CD20+ BAFFr-
C
Live Memory B cells within total B cells
% Live/Dead-CD20+ CD27+ within B cells
D
Apoptotic Memory B cells within total B cells
% Live/Dead-CD20+ CD27+BAFFr-
E
Live Naive B cells within total B cells
% Live/Dead-CD20+ CD27-CD24+CD38+ within B cells
F
Apoptotic Naive B cells within total B cells
% Live/Dead-CD20+ CD27+CD24+CD38+BAFFr-
G
Live Transitional B cells within total B cells
% Live/Dead-CD20+ CD27-CD24hiCD38hi within B cells
H
Apoptotic Transitional B cells within total B cells
% Live/Dead-CD20+ CD27+CD24hiCD38hiBAFFr-
0 5 10 15 25
Tacrolimus (ng/mL)
0 5 10 15 25
MMF (ug/mL)
0 10 50 100 500
Steroids (ng/mL)

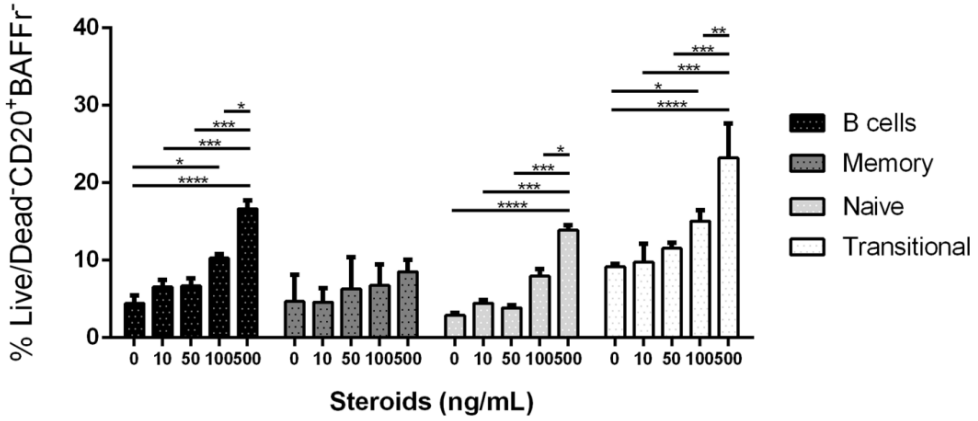
Figure 5: The effects of immunosuppressive drugs in B cell viability and apoptosis *in vitro.*

Left column shows live B cells, defined as Live/Dead^{-}CD20^{+}, and the right column shows apoptotic B cells, defined as Live/Dead^{-}CD20^{+} BAFFr^{-}. Identification of live (A) and apoptotic (B) B cells; live (C) and apoptotic (D) Memory B cells; live (E) and apoptotic (F) Naïve B cells; live (G) and apoptotic (H) Transitional B cells are shown after treatment with immunosuppressive drugs. Tacrolimus, MMF and steroids were added to PBMCs cultures for 24 hours at concentrations showed in the X axes. Live/Dead and BAFFr expression was analysed by flow cytometry. Two-way RM ANOVA test with a Tukey's multiple comparisons test was used, * $p<0.05$ was considered significant.

A


Specific B cell-death induced by Steroids
% Live/Dead-CD20+ w/o-Steroids - Live/Dead-CD20+ w-Steroids
Steroids (ng/mL)
B cells
Memory
Naive
Transitional

B


B cell apoptosis induced by Steroids
% Live/Dead-CD20+BAFFr-
Steroids (ng/mL)
B cells
Memory
Naive
Transitional

C

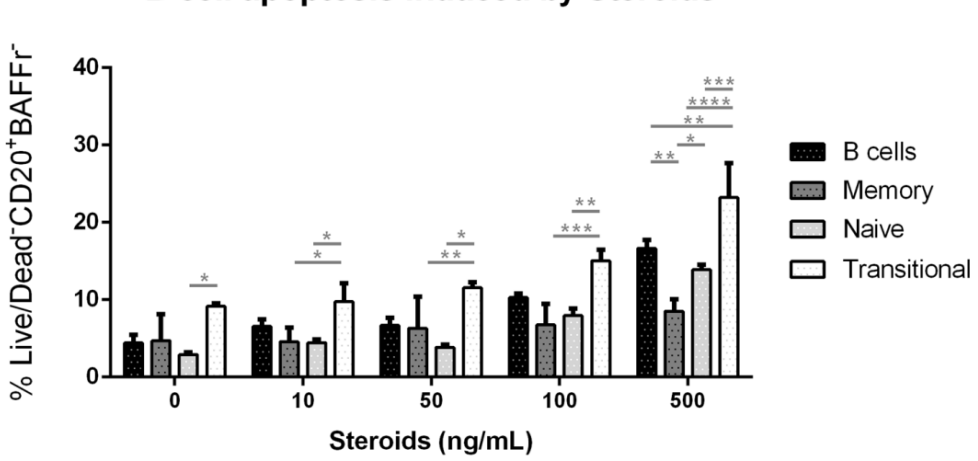

B cell apoptosis induced by Steroids
% Live/Dead-CD20+BAFFr-
Steroids (ng/mL)
B cells
Memory
Naive
Transitional

Figure 6: Steroids effect in cell viability and apoptosis in B cell subsets *in vitro*.

Cell viability in B cell subsets was measured as the difference between live B cell treated with and without different concentration of steroids (A). Apoptosis was measured as Live/Dead^{-}CD20^{+}BAFFr^{-} within B cell subsets and percentages of apoptotic cells were compared between B cell subsets (B) and between steroid concentrations (C). Two-way RM ANOVA test with a Tukey's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.2.5 Transitional B cells exhibited the highest median CD20 expression.

As previously mentioned in the introduction, *MS4A1* is the encoding gene for CD20, one of the main B cell markers. This was the only common up-regulated gene in tolerant recipients from the IOT study (Sagoo *et al*. 2010), Brouard's study (Brouard *et al*. 2007; Pallier *et al*. 2010) and the ITN study (Newell *et al*. 2010). Interestingly, Transitional B cells express higher levels of CD20, compared to Naïve and Memory B cells (Cuss *et al*. 2006). In this study, protein expression of the CD20-encoding gene*, MS4A1,* on Transitional B cells from patient groups and healthy controls was investigated.

To address if CD20 was higher in tolerant recipients and whether or not it was due to the presence of Transitional B cells, a specific flow cytometry analysis was used, in which MFI estimated the amount of CD20 molecules expressed per cell. Once this analysis was standardised, the MFI of CD20 was investigated for all B cells subsets from patient samples and healthy controls, using CD19 as a control.

It was observed that the median expression of CD20 was higher in Transitional B cells, compared to IgM^+/IgD^+ (*p=0.0391), Naïve (****p<0.0001) and Mature cells (****p<0.0001) (Fig7 A), whereas CD19 MFI was lower in Transitional B cells, compared to Memory B cells (*p=0.0120) (Fig7 B). The difference of CD20 MFI between total and Transitional B cells in each patient group was studied, and an increased CD20 MFI of Transitional B cells was observed in healthy control (****p<0.0001), tolerant (****p<0.0001), stable (*p<0.0285) and chronic rejector (***p<0.0003) (Fig7 C). Conversely, although no difference in the levels of the expression of CD19 between total and Transitional B cells was observed between patients groups, CD19 MFI from these two populations was significantly decreased in chronic rejector (*p<0.05) (Fig7 D).

Addressing a potential function of CD20 in tolerance was difficult as the role of the CD20 signalling pathway in the B cells remains unknown. There are only few reports regarding the function of this protein in humans, but none was linked to tolerance. Therefore, it was necessary to find any preliminary evidence to explain the high expression of this marker in Transitional B cells. The expression and the localisation of CD20 in the B cells subsets was studied, followed by evaluation of any

possible co-localisation with the rest of the phenotypic markers expressed by these cells. The aim was to evaluate if CD20 was interacting with more proteins, specifically in Transitional B cells, and if these potential interactions were important for Transitional B cell function and survival.

Expression and localisation of the same markers used in the B cell flow panel were this time measured by ImageStream. Results confirmed that Transitional B cells (pink dots) showed the highest CD20 expression in a CD20/CD19 dot-plot (Fig7 E), as previously observed using MFI in Figure 7A.

The distribution of CD20 was then evaluated using the delta centroid (Fig7 F). This parameter measures the difference of CD20 distribution between the cell centre and any polarised zone in the cell. Proteins equally distributed exhibit the highest fluorescent zone in the centre of the cell; therefore when markers are equally distributed, delta centroid values are close to 0. On the other hand, when markers are polarised, delta centroid increase between the cell centre and the polarised zone, and values are > 0. CD20 distribution was not polarised and the expression of this marker was equally distributed in the B cells. Delta centroid was then used to identify the location and polarisation of all B cell markers used in the B cell panel in Memory, Naïve and Transitional B cells from a healthy volunteer (Fig7 G). Although differences in the expression of the markers were observed between B cell subsets, no differences were exhibited regarding protein polarisation.

Finally, co-localisation between CD20 and B cell markers were analysed using Inspire software. An example of co-localisation between CD20 and CD24/CD38 in Transitional B cells is shown in Figure 7F. Equal distribution was found for CD20 and the rest of the B cell markers, and no specific co-localisation was observed between markers; delta centroid values for all surface markers were close to 0 (data not shown).

In conclusion, high expression of CD20*,* previously found in tolerant recipients, could be explained by the high expression of this protein found in Transitional B cells. Although Transitional B cells from all patient groups exhibited higher CD20 MFI, compared to other B subsets, percentages of Transitional cells were only augmented in tolerant patients. However, the role of CD20, if any, in tolerance remains unclear. No evidence of CD20 interaction with other surface markers was observed, the protein was equally distributed and no polarisation was found in any B cell subset.

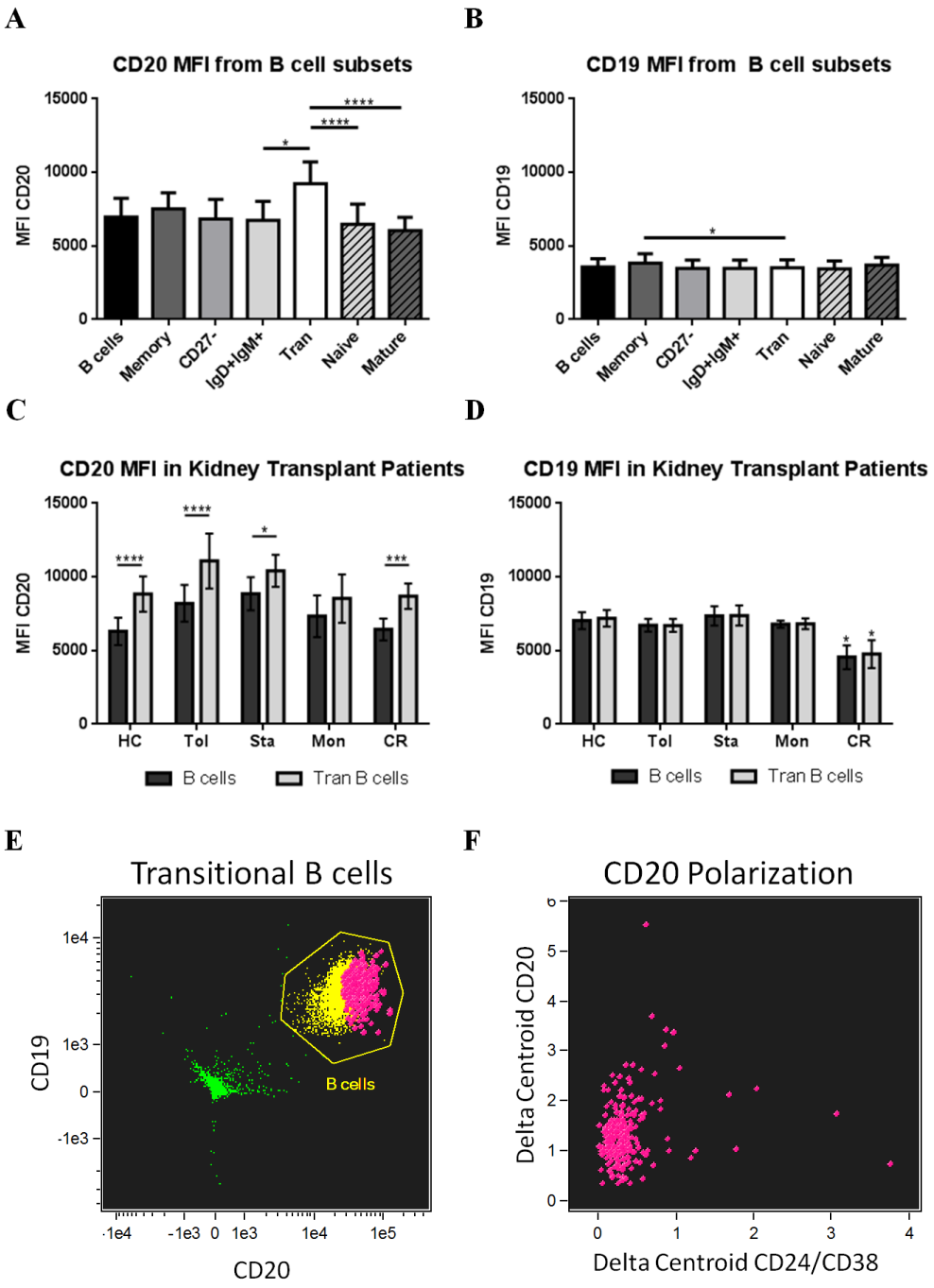

A
CD20 MFI from B cell subsets
MFI CD20
15000
10000
5000
0
B cells
Memory
CD27-
IgD+IgM+
Tran
Naive
Mature
B
CD19 MFI from B cell subsets
MFI CD19
C
CD20 MFI in Kidney Transplant Patients
HC
Tol
Sta
Mon
CR
B cells
Tran B cells
D
CD19 MFI in Kidney Transplant Patients
E
Transitional B cells
CD19
CD20
B cells
F
CD20 Polarization
Delta Centroid CD20
Delta Centroid CD24/CD38
G
Memory B cells
Naive B cells
Transitional B cells
CD20
CD27
IgM
IgD
CD24
CD38
CD24/CD38

Figure 7: CD20 and CD19 MFI.

CD20 (A) and CD19 (B) MFI was measured in B cell subsets from healthy volunteers. CD20 (C) and CD19 (D) MFI was measured in total B and Transitional B cells from healthy control (HC), tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR). Friedman test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant. Transitional B cells (pink dots) within B cells (yellow dots) were identified in a CD20/CD19 dot-plot analysed by ImageStream (E). The distribution of the CD20 molecule within the B cells was measured using delta centroid (values close to 0 demonstrate equal distribution). Co-localisation of the CD20 molecule with CD24/CD38 expression was analysed using delta centroid (values close to 0 demonstrate equal distribution) (F). The distribution of all B cell markers in Memory, Naïve and Transitional B cells were displayed in a gallery pictures obtained after ImageStream analysis (G).

4.3 Functional characterisation of B cell subsets in kidney transplant patients.

4.3.1 B cells from kidney transplant patients up-regulated CD25 and CD86 after cognate CD40 activation

Once the B cell subset distribution and phenotypic characteristics were clearly defined in each patient group, an investigation was carried out into the capacity of B cell subsets to secrete IL-10, a known key cytokine produced by regulatory B cells. However before testing this, the question of whether all the different B cell subsets could be activated by different stimuli was evaluated. CD40 activation of B cells was the first stimulus studied because this activation has been shown to induce regulatory properties in Transitional B cells by CD80/CD86 activation and IL-10 production (Blair *et al*. 2010). Firstly, the relationship between B cell activation markers and tolerance was studied, followed by the measurement of IL-10-producing B cells from the different patient groups.

Co-stimulatory molecules have different roles in B cell activation; for example after B cell activation, CD80/86 are up-regulated and induces T cell activation *via* CD28 interaction (Tu *et al*. 2008), or anergy *via* CTLA-4 (Clark *et al*. 1994). Other co-stimulatory signal is the B-T cell CD40-CD40L interaction. This activation has an important role in the development and class-switching of the B cells (Agematsu *et al*. 1998), and genetic absence of CD40L in human results in hyper IgM syndrome whereby B cells are unable to class switch to IgG (Agematsu *et al*. 1998). Another molecule up-regulated after B cell activation is the alpha-chain of the IL-2 receptor, CD25. This activation has an important role in survival signalling (Schultz *et al*. 2001). For all these reasons, the focus of the study moved towards CD86, CD40 and CD25 expression on B cell subsets.

Since CD40 stimulation was critical for the regulatory B cell activation (Duddy *et al*. 2004; Jamin *et al*. 2008), different methods were used to obtain optimal CD40 activation. In a first attempt, soluble anti-CD40 antibody was tested to emulate CD40-CD40L interaction, but no evidence of B cell activation was observed (data not shown). In a second attempt, L cells transfected with CD40L were used to replicate the interaction between CD40 and CD40L. These cells were kindly donated by Dr. Case

Van Kooten to Professor Giovanna Lombardi. CD40L expression was evaluated in both non-transfected and CD40L-transfected L cells, using an anti-CD40L antibody. Histogram showed a positive CD40L expression in transfected cells, whereas no positive signal was identified in non-transfected cells (Fig8 A).

Up-regulation of co-stimulatory molecules in $CD20^{+}$ cells was analysed to ensure B cell activation after stimulation with CD40L-transfected cells. CD86 and CD25 expression was measured in B cells within PBMCs. Histogram showed an up-regulation of CD86 and CD25 after CD40-mediated B cell activation in a healthy volunteer (Fig8 B).

Once the expression of CD25 and CD86 molecules were correctly up-regulated after cell activation in healthy volunteers, the B cell response in samples from kidney transplant patients, compared to results from healthy controls was evaluated. To perform the experiments, patient samples were activated with CD40L-transfected L cells, using non-transfected L cells as a control.

Expression of CD25 and CD86 molecules were measured in total B cells from healthy controls and kidney transplant patients. The first findings demonstrated that B cells from all groups up-regulated CD25 (Fig8 C) and CD86 (Fig8 D) molecules following CD40 activation. These results suggest that B cells from all patient groups were capable to respond to CD40-CD40L interaction, and that immunosuppressive drugs did not affect the B cell response after activation. Although a significant difference was observed for CD25 up-regulation in tolerant patients compared to patients in monotherapy (*p=0.0138), no significant differences were observed in the expression of CD86 molecule between patient groups. Because B cell activation in tolerant recipients did not display clear differences in the expression of this molecule compared to other patient groups, it is difficult to support the idea that up-regulation of CD86 is linked to transplantation tolerance.

Expression of CD25 (Fig8 E) and CD86 (Fig8 F) were then measured in Memory B cells from healthy controls and kidney transplant patients. Although activation in this subset was ascertained by up-regulation of both molecules in all patients groups after CD40 activation, no significant differences in activated B cells were observed between groups.

Finally, expression of CD25 (Fig8 G) and CD86 (Fig8 H) was measured in $CD27^-$ B cells (Naïve and Transitional), and again, CD25 and CD86 were up-regulated in B cells from all patients groups after CD40 activation. No significant differences in percentages $CD86^+$ B cells were found between groups, however, CD25 was differentially expressed in B cells from tolerant patients compared to patients in monotherapy (*p=0.024) and chronic rejector (*p=0.040). These differences were also observed between the last groups and healthy controls.

CD40, the protein that interacts with CD40L, was also measured in total B cells (Fig9 A), Memory (Fig9 B) and $CD27^-$ (Fig9 C) B cells, but no significant differences were found between activated samples or between groups.

In conclusion, up-regulation of CD86 was not specific for tolerant recipients and activation of B cell responses after CD40 ligation was observed in all patient groups, independently of the immunosuppressive drug treatment. CD25 expression was specifically increased in CD40-activated $CD27^-$ B cells from tolerant patients; following on from these results, it would not be far-fetched to suggest that when CD25 is up-regulated in these cells, there may be an increase in IL-2 capture, as CD25 is part of the IL-2 receptor. Taking this into consideration, if the IL-2 signalling pathway is triggered in activated Naïve and Transitional B cells, survival genes will be transcribed. If this does occur, cells from tolerant recipients will respond better to CD40 activation than B cells from other patient groups, and will therefore secrete more IL-10 as they support better this particular activation.

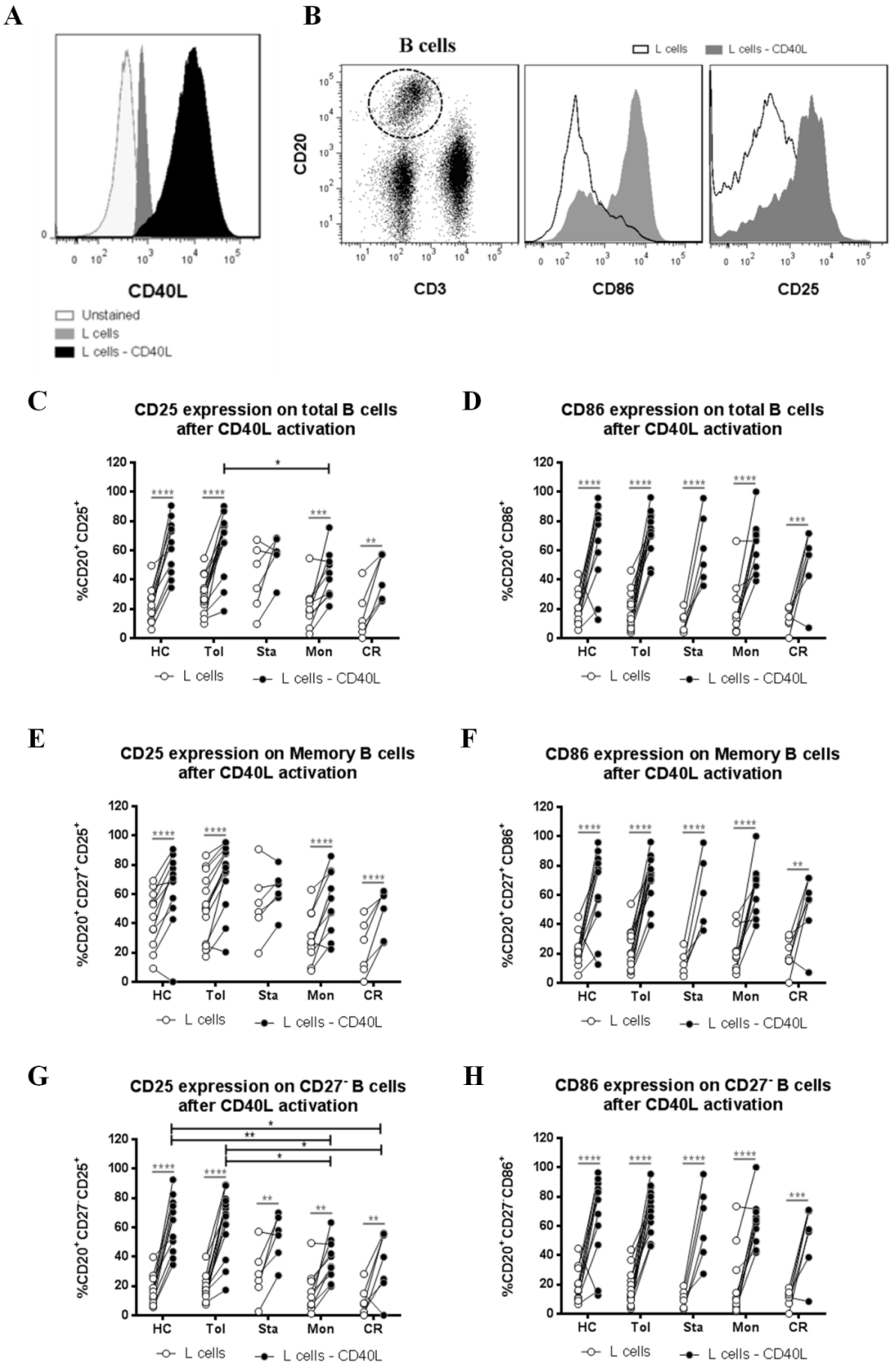
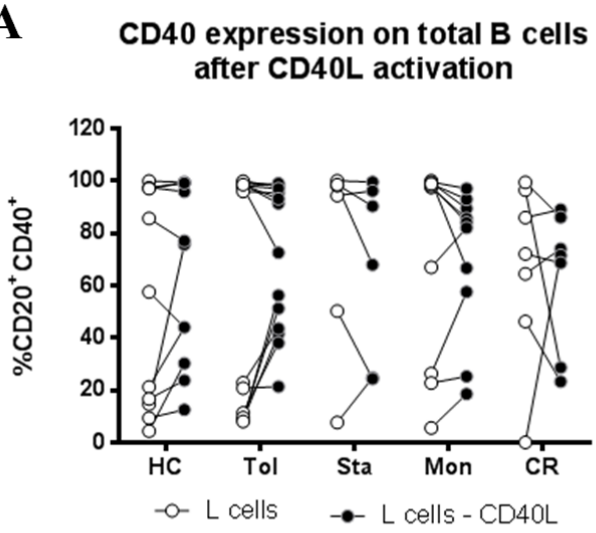

A
CD40L
Unstained
L cells
L cells - CD40L
B
B cells
L cells
L cells - CD40L
CD20
CD3
CD86
CD25
C
CD25 expression on total B cells after CD40L activation
%CD20+CD25+
D
CD86 expression on total B cells after CD40L activation
%CD20+CD86+
E
CD25 expression on Memory B cells after CD40L activation
%CD20+CD27+CD25+
F
CD86 expression on Memory B cells after CD40L activation
%CD20+CD27+CD86+
G
CD25 expression on CD27- B cells after CD40L activation
%CD20+CD27-CD25+
H
CD86 expression on CD27- B cells after CD40L activation
%CD20+CD27-CD86+
HC
Tol
Sta
Mon
CR
L cells
L cells - CD40L

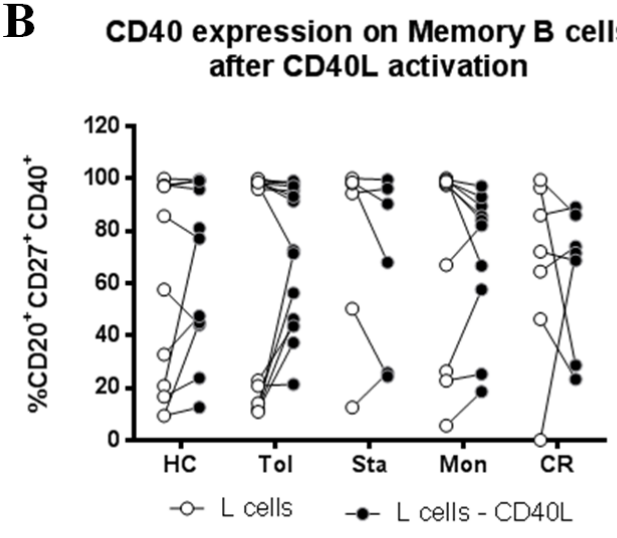
Figure 8: Expression of activation molecules in kidney transplant patients.

The expression of CD40L was measured in $1.0x10^6$ unstained CD40L-transfected L cells□, CD40L-PE-stained non-transfected L cells ■ and CD40L-PE-stained CD40L-transfected L cells ■ (A). The expression of CD86 and CD25 molecules on $CD20^+$ B cells was measured by surface staining after 3 days of activation with $0.5x10^5$ non-transfected L cells □ and CD40L-transfected L cells ■ (plate-bound) (B). Expression of CD25 (C) and CD86 (D) from total B cells, expression of CD25 (E) and CD86 (F) from Memory B cells and expression of CD25 (G) and CD86 (H) from $CD27^-$ B cells was measured by surface staining in $1.0x10^6$ PBMCs from healthy control (HC) tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR) after 3 days of activation with $0.5x10^5$ non-transfected L cells and CD40L-transfected L cells (plate-bound). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used; * $p<0.05$ was considered significant.

A


CD40 expression on total B cells
after CD40L activation
%CD20+CD40+
120
100
80
60
40
20
0
HC
Tol
Sta
Mon
CR
L cells
L cells - CD40L

B


CD40 expression on Memory B cells
after CD40L activation
%CD20+CD27+CD40+
120
100
80
60
40
20
0
HC
Tol
Sta
Mon
CR
L cells
L cells - CD40L

C

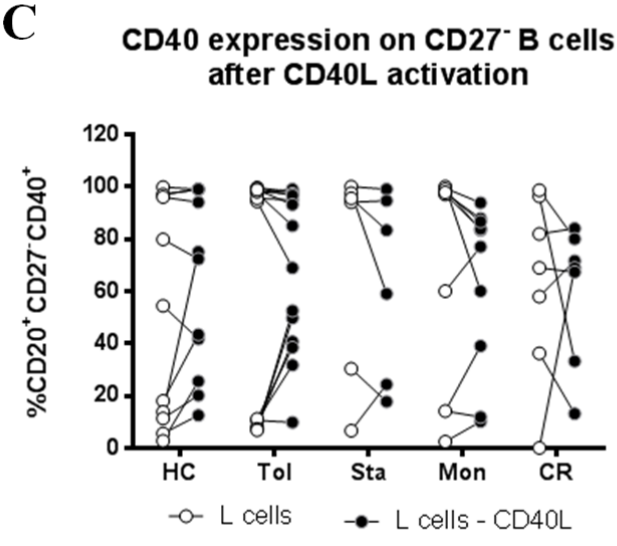

CD40 expression on CD27- B cells
after CD40L activation
%CD20+CD27-CD40+
120
100
80
60
40
20
0
HC
Tol
Sta
Mon
CR
L cells
L cells - CD40L

Figure 9: Expression of CD40 on B cell subsets.

Expression of CD40 from total B cells (A), Memory B cells (B) and $CD27^-$ B cells (C) was measured by surface staining in $1.0x10^6$ PBMCs from healthy control (HC) tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR) after 3 days of activation with $0.5x10^5$ non-transfected L cells and CD40L-transfected L cells (plate-bound). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.3.2 B cells from kidney transplant patients up-regulated TLR-9 after CpG activation

The second stimulus used to activate B cells was CpG, cytosine-guanine DNA regions (Liang *et al*. 2010). CpG, like CD40 ligation, has previously been shown to induce optimal stimulation of IL-10 production by total B cells (Liang *et al*. 2010). This activation occurs through TLR-9, an intracellular toll like receptor that recognises specific unmethylated CpG oligonucleotide (ODN) sequences from microbial DNA.

CpG-TLR-9 activation was used to ensure that the IL-10 signalling pathway was not affected in recipients by the effect of immunosuppressive drugs, and since CD40 activation preferentially targeted Transitional B cells, it was also used to induce a regulatory response from all the B subsets.

Since up-regulation of CD86 and CD25 molecules confirmed CD40 activation (prior to IL-10 secretion), TLR-9 expression was used to confirm CpG activation. Thus, TLR-9 expression was studied in unstimulated and CpG-stimulated samples from healthy controls and kidney transplant patients using intracellular staining. Histograms (Fig10 A) and dot-plots (Fig10 B) showed an increase of TLR-9 expression in CpG-activated B cells (blue) compared to unstimulated B cells (black).

TLR-9 expression was then measured in B cells from kidney transplant patients and healthy controls, with and without CpG activation (Fig10 C). Differences in TLR-9 expression, between non-activated and activated samples, were observed in B cells from healthy controls (**p=0.0062), tolerant (**p=0.0015), stable (*p=0.0460) and monotherapy (**p=0.0060) patients. No difference between CpG-activated samples between groups was observed.

B cells from all patient groups, except chronic rejector, responded to CpG activation *via* TLR-9 expression. Interestingly, because chronic rejector was the only group not responding to this stimulus, it was important to determine in the following experiments whether IL-10 production was also affected. Finally, because stable and monotherapy patients up-regulated TLR-9 expression, the study confirmed that immunosuppressant drugs were not affecting the CpG-TLR-9 activation on the B cells.

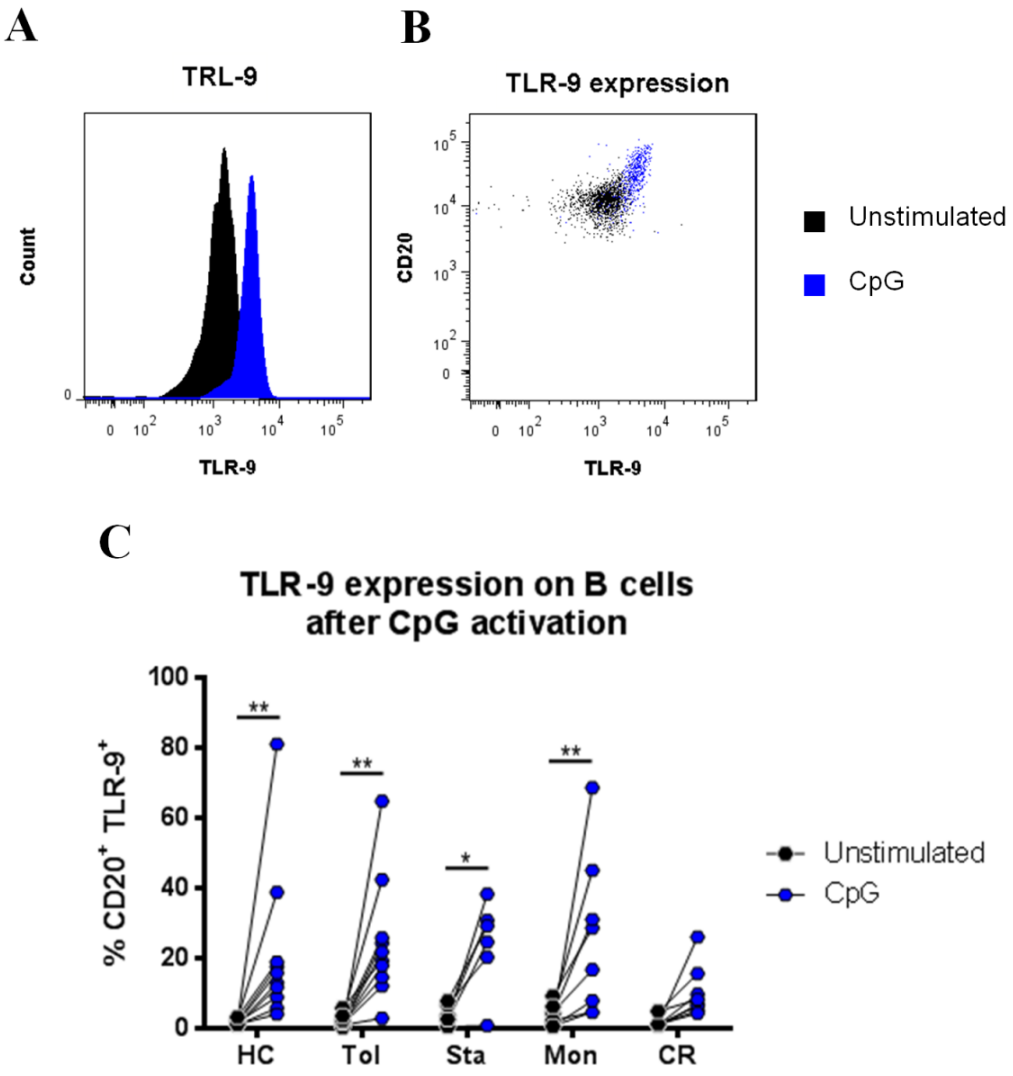


Figure 10: Expression of TLR-9 after CpG activation.

Expression of TLR-9 from B cells was measured in unstimulated ■ and 1uM CpG-stimulated ■ samples using a histogram (A) or a dot-plot (B) after 3 days of culture. Expression of TLR-9 from unstimulated ⬢ and 1μM CpG-stimulated ⬢ B cells was measured by intracellular staining in $1.0x10^6$ PBMCs from healthy control (HC) tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR) samples after 3 days of culture (C). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.3.3 Transitional B cells produced the highest levels of IL-10 after CD40 activation

Having demonstrated that in transplant recipients and healthy controls, ligation of CD40 and CpG up-regulated activation markers and TLR-9, respectively, a study into whether the same stimuli can induce IL-10 production by B cells was undertaken. The focus of the study was on IL-10 production due to the important role this cytokine plays in immune regulation (Blair *et al*. 2010; Liang *et al*. 2010; Iwata *et al*. 2011).

Particularly relevant to this study, Transitional B cells have been shown to control the immune responses through IL-10 production. (Blair *et al*. 2010). Claudia Mauri's group revealed a down-regulation of the Th1 response and observed lower percentages of $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD4^{+}TNF\text{-}\alpha^{+}$ cells when Transitional B cells were present in culture. This regulatory property was diminished when the IL-10 pathway was blocked, revealing that IL-10 was one of the mechanisms of immune regulation used by these cells. In this study, tolerant patients exhibited higher percentages of Transitional B cells than other patient groups. IL-10 production after CD40 activation was then studied, to understand if Transitional B cells were participating in tolerance through IL-10 regulation. Since CD40 activation targeted mainly Naïve and Transitional B cells (Duddy *et al*. 2004; Duddy *et al*. 2007; Blair *et al*. 2010), the potential regulatory role of Memory B cells in tolerant transplant patients was evaluated using CpG stimulation.

IL-10 production was measured in kidney transplant patients by intracellular staining and ELISA. The first method allowed the measurement of IL-10 specifically produced by B cells, and the second allowed the measurement of total levels of cytokine after three days of culture. Both methods were evaluated in PBMCs and isolated B cells, but only PBMCs were used due to the difficulty to isolate B cells from patient samples and the decrease of cell viability observed when isolated B cells were cultured.

Intracellular staining was used to identify IL-10-producing cells, especially in the Transitional population. However, this approach was not optimal in the identification of the different B cell subsets. Unfortunately, after three days of culture and the use of PMA and ionomycin, the high expression of CD24 and CD38 was

completely lost, therefore the identification of Transitional B cells by this method was not possible. Because of this, sorted Memory, Naïve and Transitional B cells from healthy volunteers were used, to determine how CD40 and CpG activation were affecting each B cell subset, and to then apply this principle to patient samples. IL-10 production in CD40 or CpG-activated B cell subsets was measured in the form of $CD20^{+}IL\text{-}10^{+}$ cells, using intracellular staining (Fig11 A) and ELISA.

The results obtained demonstrated that CD40-activated Transitional B cells triggered a specific and distinctive IL-10 response. After three days of culture, an increment of $CD20^{+}IL\text{-}10^{+}$ cells was mainly observed in the Transitional population (**p=0.0049) after CD40 activation (Fig11 B). Although IL-10 secretion was also observed in the Transitional population using ELISA, no significant differences were found between non-activated and CD40-activated samples (Fig11 C).

Conversely, the Memory B cell subset was the main population targeted by CpG activation. Although $IL\text{-}10^{+}$ cells were increased in Memory (****p>0.0001), Naïve (**p=0.0025) and Transitional (***p=0.0005) B cells (Fig11 D) after CpG activation, protein levels were only significantly increased in the supernatants from Memory B cells (**p=0.0076) (Fig11 E). Altogether CpG-activated Memory B cells were the higher responders compared with the other two subsets, shown by intracellular staining and ELISA.

From these results, it was concluded that CD40 activation targets mainly Transitional B cells, whereas CpG activation targets mainly Memory B cells.

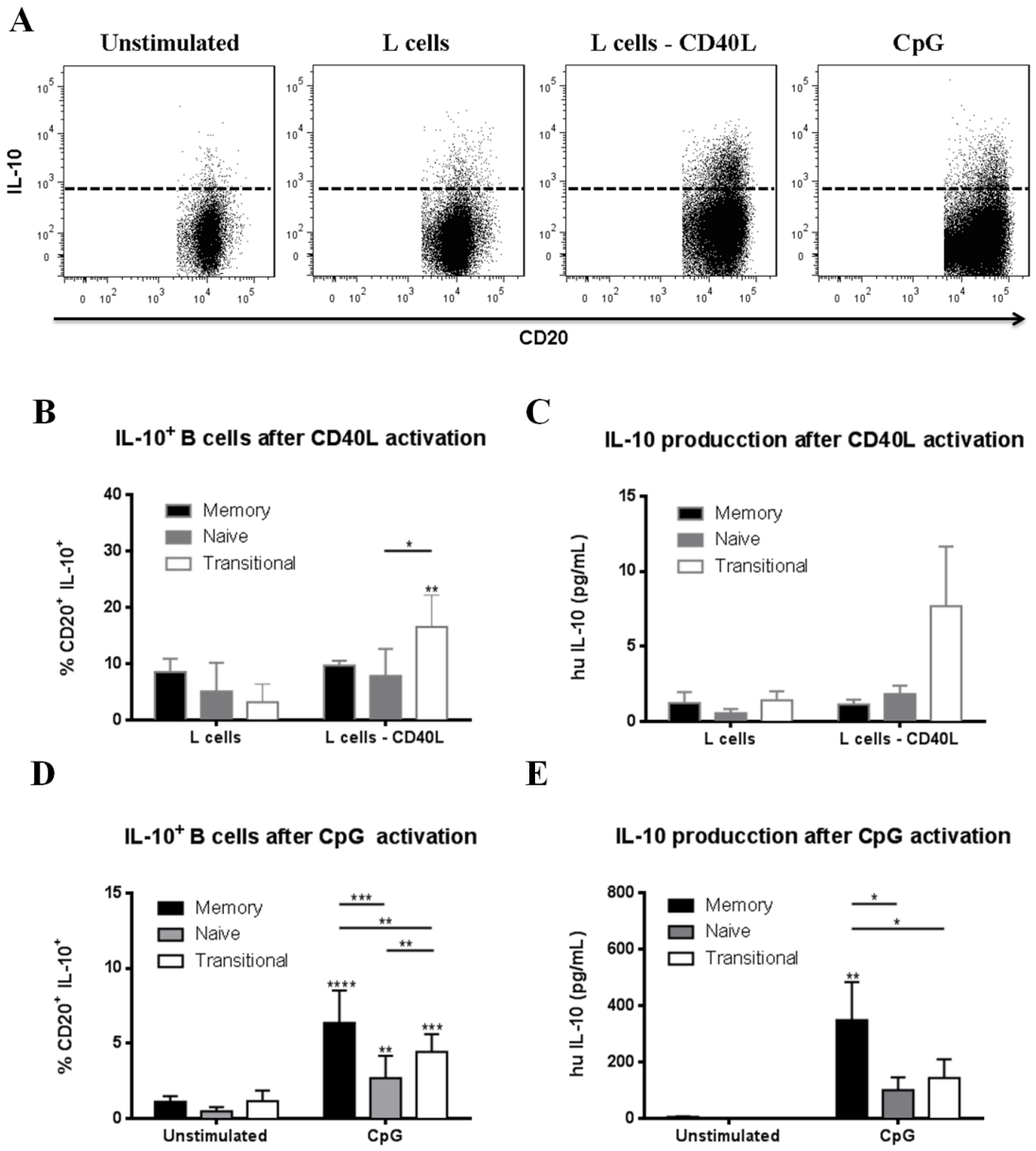


Figure 11: IL-10 production by B cell subsets from healthy volunteers (cones).

Sorted B cell subsets were stimulated with and without $1.0x10^4$ non-transfected L cells, $1.0x10^4$ CD40L-transfected L cells (plate-bound), or 1uM CpG during 3 days, and representative dot-plots of $CD20^+IL\text{-}10^+$ B cells were displayed to demonstrate the quality of the intracellular staining (A). IL-10 was measured in cultures of $0.5x10^6$ B cell subsets stimulated with $1.0x10^4$ non-transfected L cells, or $1.0x10^4$ CD40L-transfected L cells (plate-bound) after 3 days using intracellular staining (B) and ELISA (C). IL-10 was also measured in cultures of $0.5x10^6$ B cell subsets stimulated with or without 1uM CpG after 3 days using intracellular staining (D) and ELISA (E). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.3.4 B cells from tolerant recipients produced higher levels of IL-10 and lower levels of TNF-α than B cells from chronic rejector

IL-10 production was measured in CD40- or CpG-activated samples from kidney transplant patients and healthy controls. The aim of this part of the study was to determine if IL-10 was differentially produced by B cells from tolerant recipients.

To address this question, PBMCs from patients and healthy controls were stimulated for three days with and without non-transfected L cells, CD40L-transfected L cells or CpG. Although the cytokine of most interest evaluated was IL-10, TNF-α production was also analysed to evaluate whether this pro-inflammatory cytokines was also secreted during the two activations methods. Whereas intracellular staining was used to identify IL-10-producing B cells, ELISA was used to measure IL-10 and TNF-α in supernatants. Results were analysed with two multiple comparisons. The first multiple comparison test analysed significant differences between non-activated and CD40- or CpG-activated samples in each patient group, and the second multiple comparison test analysed significant differences in CD40- or CpG-activated samples between patient groups. For a better understanding, and because both statistical analysis are displayed in the graphs, first analysis is shown in grey and second analysis is shown in black.

IL-10 expression by B cells (Fig12 A), and IL-10 production in the supernatants (Fig12 B) were measured in CD40-activated patient samples. Although differences in $CD20^{+}IL\text{-}10^{+}$ cells between non-activated and activated samples were observed only in tolerant patients (****p>0.0001) and healthy controls (**p=0.0111), protein levels were significantly increased in all groups except chronic rejector. These findings revealed that IL-10-producing B cells were mainly present in groups with high percentages of Transitional B cells. They also suggested that immunosuppressive drugs did not block IL-10 production in general, as IL-10 was observed in supernatants from stable and monotheraphy patients. Results also suggest that IL-10 production is affected in rejection as this cytokine was not significantly different in CD40-activated B cells or in the supernatant from chronic rejector. Differences between patient groups were analysed as well, and it was observed that $CD20^{+}IL\text{-}10^{+}$ cells B cells were increased in tolerant patients compared to patients in monotherapy (**p=0.023) and chronic rejector (**p=0.0080) at day 3, but no differences between groups were observed when IL-10

was measured in supernatants. Again this result supports the idea that B cells from tolerant recipients were the main IL-10 producing-cells.

At the same time, CpG activation was studied in transplant patients and healthy controls. The percentages of $CD20^{+}IL\text{-}10^{+}$ B cells (Fig12 C), and IL-10 levels in the supernatant (Fig12 D) were measured in non-activated and CpG-activated PBMCs. Unlike CD40L, differences in IL-10 production by B cells, between non-activated and activated samples, were observed in all patients groups, except chronic rejector. However, IL-10 levels in supernatants were increased in tolerant patients (***p=0.0006) and chronic rejector (*p=0.0145). In conclusion and similar to CD40 activation, B cells from tolerant recipients exhibited high expressions of IL-10 in CpG-activated samples. However, it is still unknown whether the high-levels of IL-10 observed in some supernatants from chronic rejector, came specifically from B cells, as no increase of $CD20^{+}CD10^{+}$ cells was observed after activation in this group compared to tolerant (***p=0.0006) and stable (*p=0.0452) patients. Because of this, it is possible that other PBMCs were participating in IL-10 secretion from the chronic rejector samples.

Finally, TNF-α secretion was measured in supernatants from transplant patients and healthy controls after 3 days of activation with CD40L (Fig12 E) or CpG (Fig12 F). Only samples from chronic rejector had significantly increased levels of TNF-α after CD40L (**p=0.0013) and CpG (*p=0.0113) activation.

As a general conclusion it can be stated that B cells from tolerant recipients are the main IL-10 producing cells, independent of the type of activation. CD40 activation targeted Transitional B cells; therefore groups with high percentages of these cells exhibited high percentages of $CD20^{+}IL\text{-}10^{+}$ cells. Although healthy controls and tolerant patients were the main CD40L responders, B cells from other patients groups also responded to CD40 activation with IL-10 production, but at lower levels. This means that IL-10 is not impaired in non-tolerant recipients; hence, results suggest that differences in $CD20^{+}IL\text{-}10^{+}$ percentages are directly proportional to Transitional B cells percentages found in patient samples. In addition, it is suggested that immunosuppressive drugs were not blocking the IL-10 signalling pathway in B cells.

Memory B cells were the predominant responder B cell population activated by CpG. All groups exhibited high percentages of $CD20^{+}IL\text{-}10^{+}$ cells, and no differences in

percentages were found between patient samples after activation, except in chronic rejector. Because of this, it can be stated that CpG activation affected equally all B subsets from all patient groups, expect chronic rejector.

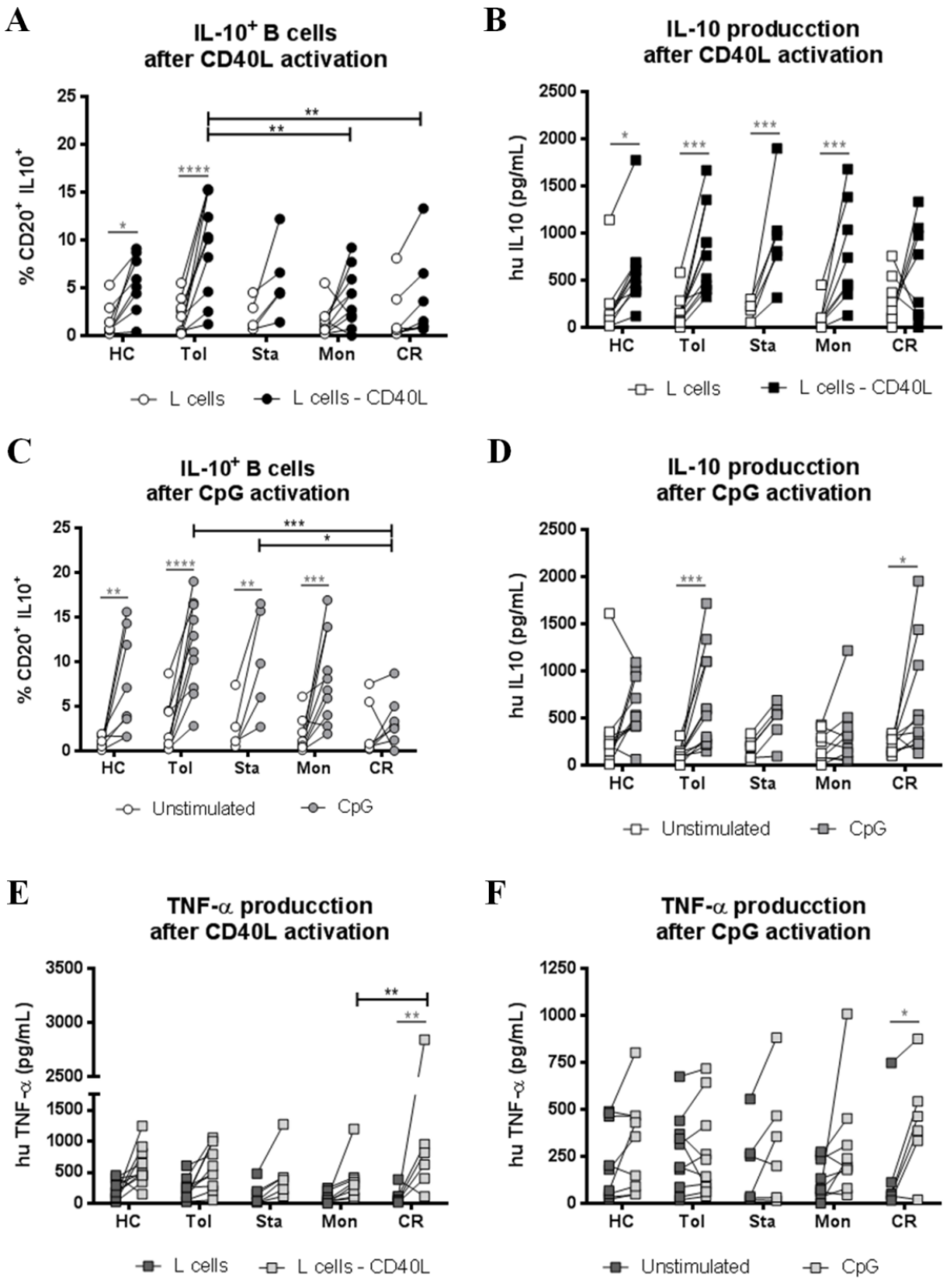


Figure 12: IL-10 production by B cell subsets from kidney transplant recipients

IL-10 and TNF-α production was measured in CD40- and CpG-activated PBMCs from healthy control (HC), tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR). $1.0x10^6$ PBMCs were stimulated with $0.5x10^5$ non-transfected L cells or $0.5x10^5$ CD40L-transfected L cells (plate-bound), and after 3 days of culture, IL-10^+Bcells (A) and secreted IL-10 in supernatants (B) were measured by intracellular staining and ELISA, respectively. PBMCs were also activated with and without 1uM CpG during 3 days of culture. IL-10^+Bcells (C) and secreted IL-10 in supernatants (D) were measured by

intracellular staining and ELISA, respectively. Finally, levels of TNF-α in the supernatants of $1.0x10^6$ PBMCs activated with $0.5x10^5$ non-transfected L cells or $0.5x10^5$ CD40L-transfected L cells (plate-bound) (E), and with or without 1uM CpG (F) were measured after 3 days of culture. Two-way RM ANOVA test with a Sidak's multiple comparisons test was used, * $p<0.05$ was considered significant.

4.3.5 "Loss Tolerance" kidney transplant recipients reduced Transitional B cells and the capacity to produce IL-10 over time

If IL-10 is participating in the process of transplantation tolerance, patients that lost their tolerant state should display alterations in this pathway over time. Three "Loss Tolerance" patients were recruited from Sheffield, Czech Republic and Switzerland at three time points separated between six months. Sample quality was not optimal due to long distance and period of sample transportation. Cells were stained with the B cell panel (Fig1) and changes in the B cell subsets were studied over time. Loss Tolerance recipients presented different percentages of $CD20^{+}CD19^{+}$ B cells, but the percentages were not affected over time (Fig13 A). In terms of the Memory B cells, whereas all patients maintained invariable Memory B cell percentages between Time Point 1 and 2, two of three exhibited an increase in the number of Memory B cells at Time point 3 (Fig13 B). Percentages of Naïve B cells did not show any particular changes over time (Fig13 C). Interestingly, Transitional B cells from all patients decreased over time (Fig13 D). Examples of this loss of Transitional B cells are shown in Dot plots (Fig13 E).

Samples activated with CD40L (Fig13 F&H) and CpG (Fig13 G&I) were tested for IL-10 and TNF-α production using intracellular staining and ELISA. Both techniques were performed to identify all cytokines, but only ELISA was useful in determining cytokine production because poor cell quality resulted in low numbers of PBMCs after intracellular staining. Although B cells from two patients increased IL-10 production after CD40 and CpG activation, levels of IL-10 were very low compared to activated-samples from tolerant patients (Fig13 F&G). A similar result was observed with TNF-α after CD40L activation. No particular changes were observed in TNF-α production after CpG activation.

In summary, in samples from patients that lost tolerance, the percentages of Transitional B cells and the ability to produce IL-10 after CD40 and CpG activation was lost over time.

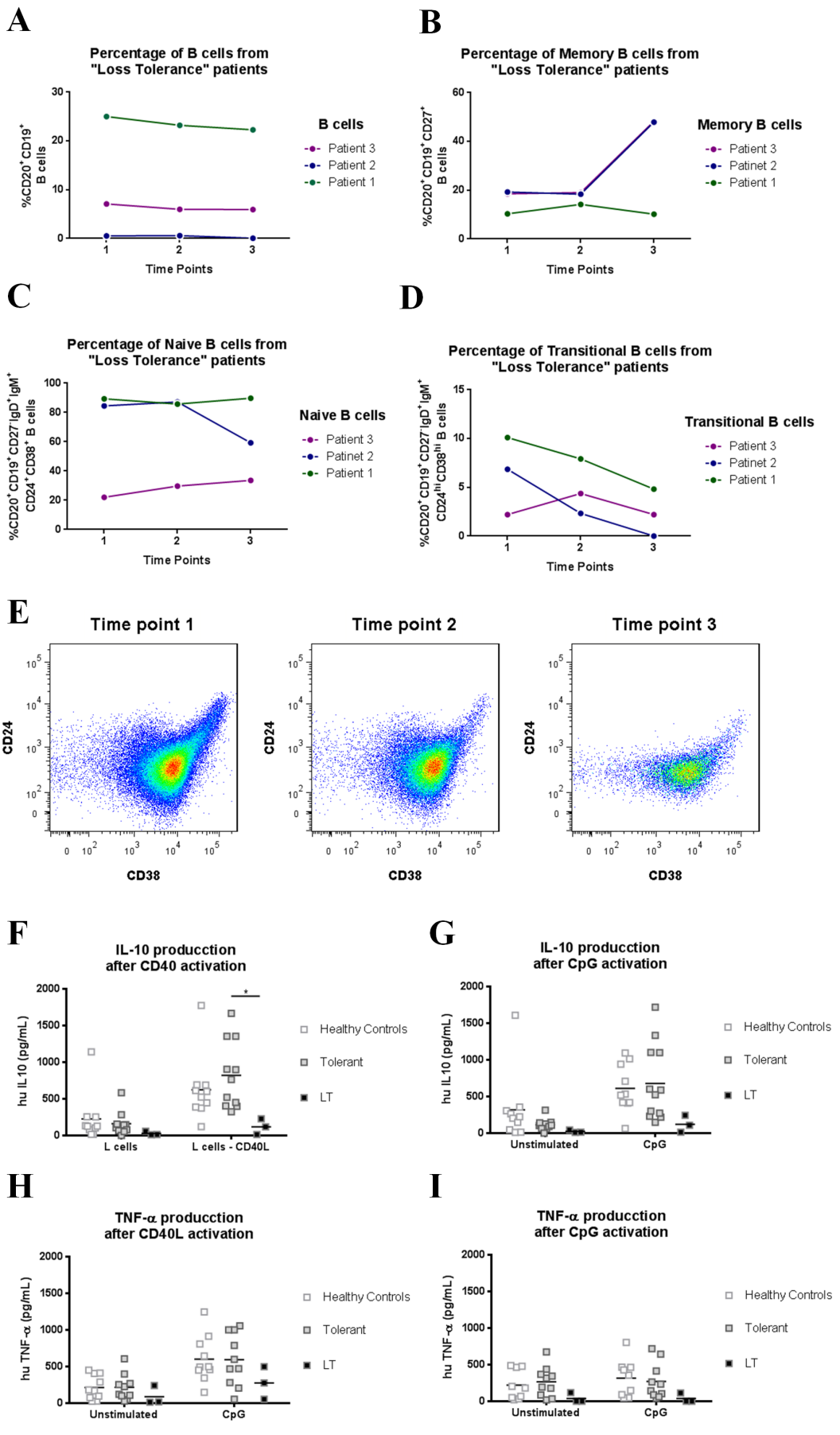

A
Percentage of B cells from "Loss Tolerance" patients
%CD20+CD19+ B cells
Time Points
B cells
Patient 3
Patient 2
Patient 1
B
Percentage of Memory B cells from "Loss Tolerance" patients
%CD20+CD19+CD27+ B cells
Time Points
Memory B cells
Patient 3
Patinet 2
Patient 1
C
Percentage of Naive B cells from "Loss Tolerance" patients
%CD20+CD19+CD27-IgD+IgM+ CD24+CD38+ B cells
Time Points
Naive B cells
Patient 3
Patinet 2
Patient 1
D
Percentage of Transitional B cells from "Loss Tolerance" patients
%CD20+CD19+CD27-IgD+IgM+ CD24hiCD38hi B cells
Time Points
Transitional B cells
Patient 3
Patinet 2
Patient 1
E
Time point 1
Time point 2
Time point 3
CD24
CD38
F
IL-10 producction after CD40 activation
hu IL10 (pg/mL)
L cells
L cells - CD40L
Healthy Controls
Tolerant
LT
G
IL-10 producction after CpG activation
hu IL10 (pg/mL)
Unstimulated
CpG
Healthy Controls
Tolerant
LT
H
TNF-α producction after CD40L activation
hu TNF-α (pg/mL)
Unstimulated
CpG
Healthy Controls
Tolerant
LT
I
TNF-α producction after CpG activation
hu TNF-α (pg/mL)
Unstimulated
CpG
Healthy Controls
Tolerant
LT

Figure 13: "Loss Tolerance" kidney transplant recipients.

Percentages of total B cells (A), Memory (B), Naïve (C) and Transitional B cells (D) were measured in "Loss Tolerance" kidney transplant recipients by surface staining using panel described in Figure 1. Examples of dot-plots of Transitional B cells at time point 1, 2 and 3 from one "Loss Tolerance" kidney transplant recipient (E). Secretion of IL-10 was measured in cultures of $1.0x10^6$ PBMCs from "Loss Tolerance" kidney transplant recipients, stimulated with $0.5x10^5$ non-transfected L cells or $0.5x10^5$ CD40L-transfected L cells (plate-bound) (F), and with or without 1uM CpG (G), after 3 days of culture using ELISA. Secretion of TNF-α was measured in cultures of $1.0x10^6$ PBMCs from "Loss Tolerance" kidney transplant recipients, stimulated with $0.5x10^5$ non-transfected L cells or $0.5x10^5$ CD40L-transfected L cells (plate-bound) (H), and with or without 1uM CpG (I), after 3 days of culture using ELISA. Wilcoxon matched-pairs signed rank test was used; * $p<0.05$ was considered significant.

4.4 Discussion.

4.4.1 Overview

The phenotypic and functional characterisation of the B cell subsets in kidney transplant recipients studied in this thesis allowed a better understanding of the role of B cells in transplantation tolerance.

The link between Transitional B cells and transplantation tolerance is based on the data showing that tolerant patients exhibited the highest percentages of Transitional B cells compared to the other patient groups maintained with immunosuppressive drugs. Because immunosuppressive drugs were the main difference between non-tolerant and tolerant recipients, the effects (specific induction of apoptosis and cell death) of tacrolimus, MMF and steroids on B cells were studied. Then, the phenotype of B cells was studied, and Transitional B cells were found to exhibit the highest expression of CD20. After phenotypic characterisation, B cell activation was studied measuring up-regulation of activation markers and cytokine production in B cells from different groups of kidney transplant patients.

4.4.2 B cell phenotype and the identification of the B cell subsets

The distribution of B cell subsets was studied in peripheral blood to identify mainly the Transitional population within PBMCs. The B cell panel used in this study was more accurate than the flow panel used by the ITN and the IOT (Newell *et al*. 2010; Sagoo *et al*. 2010), and was designed based on data from Sims and colleagues (Sims *et al*. 2005; Palanichamy *et al*. 2009).

The B cell flow panel was optimised to obtain the best phenotypic characterisation. A double positive staining using CD20 and CD19 was performed to identify total B cells. Although CD19 and CD20 are both markers fully expressed on all B cells, initial results demonstrated that discrimination of B cells using only CD19 was quite poor. On the other hand, CD20 exhibited a better separation of $CD20^{+}$ cells from non-B cells, but because Transitional B cells were the main point of interest, the subset selection strategy used was very stringent and it was decided the study would start with a double staining to avoid any possible contamination with non-B cells in the further

downstream analysis.

Once B cells were identified, CD27 was used to separate Memory B cells. Despite the fact that $CD27^-$ B cells are smaller with very little cytoplasm, and $CD27^+$ B cells are larger cells with a more abundant cytoplasm (Agematsu *et al*. 1997; Nagumo *et al*. 1998), there are not many markers that help to differentiate one from the other; CD27 was the best available marker found. Published data support the idea that CD27 is present only in Memory B cells because $CD27^+$, but not $CD27^-$ B cells, are rapidly activated and can produce higher levels of IgA, IgM, IgG and IgG subclasses (Agematsu *et al*. 1997). Another study demonstrated that $CD27^+$ B cells were absent in adult patients suffering from X-linked hyper-IgM syndrome (Agematsu *et al*. 1998). The only definitive marker of Memory B cells is the presence of somatically mutated high-affinity antigen receptors. Single-cell studies performed demonstrated that the majority of IgD^+CD27^+ B cells carry mutated V-region genes in comparison with IgD^+CD27^- B cells (Klein *et al*. 1998). Contrasting data reported the existence of $CD27^-IgG^+$ B cells in peripheral blood, but the low frequency of somatic mutation found in these cells suggests that they could represent just a first wave of Memory B cells (Fecteau *et al*. 2006). In summary, CD27 was the best option for Memory B cell discrimination.

After identification of Memory B cells as $CD27^+$, the characterisation continued from the $CD27^-$ population with the selection of double positive IgD^+IgM^+ cells. Based on previous studies that described high expression of both surface immunoglobulins in Transitional B cells, a double staining was used to improve the identification of these cells by phenotypic enrichment (Palanichamy *et al*. 2009).

Finally, Transitional and Naïve B cells were identified from the IgD^+IgM^+ population using CD24 and CD38. The selection of CD24 and CD38 was based on characterisation studies of Transitional B cells in human samples (Cuss *et al*. 2006; Palanichamy *et al*. 2009; Blair *et al*. 2010). Other important surface markers such as CD10, CD5 and CD1d (Lee *et al*. 2009; Yanaba *et al*. 2009) were not included since the aim of this characterisation was to identify of Transitional B cells within PBMCs from patient samples rather than a full phenotypic characterisation of these cells. Transitional B cells were then identified as $CD24^{hi}CD38^{hi}$, and Naïve B cells were identified as $CD24^+CD38^+$. The percentage of Naïve B cells was always the highest within the B cell

subsets (50-80%), while the percentage of Transitional B cell was the lowest (10% within B cells).

The population identified as $CD24^{+}CD38^{-}$ "Mature" B cells was an interesting population because it was mainly found in patient samples rather than in healthy controls. One explanation could be that because Memory B cells are localised in the same zone in the flow analysis, "Mature" cells could be Memory B cells with a low or absent expression of CD27. The terminology "Mature" was used because B cells were positive for IgD and IgM and thus maturation process was implicit, but also to distinguish this population from the Naïve and Transitional population (as CD24 and CD38 expression was different as well). This terminology did not imply that Naïve and Transitional B cells are not part of the mature repertoire of B cells. Finally, pre-Plasma cell precursors identified as $CD20^{-}CD19^{+}CD27^{+}CD38^{hi}$ (Harada *et al*. 1996; Arce *et al*. 2001) was a small population predominant in some patient, but absent in healthy controls.

In summary, the B cell panel designed allowed the identification of all the relevant B cells subsets present in peripheral blood (Sims *et al*. 2005). The staining of the flow panel was maintained stable during the three years of the study using CS&T beads, application settings (control for the flow cytometer variation) and an internal sample (control the surface staining/cell preparation variation).

4.4.3 B cell subset distribution observed in samples from peripheral blood, spleen and lymph nodes

In order to understand the role of Transitional B cells in tolerance, the location of these cells was identified in different human tissues associated with an immunological function.

Transitional B cells are the first population that emerge from the bone marrow into peripheral blood. Several groups have described a full phenotype in both tissues demonstrating how the B cells undergo a maturation process (Sims *et al*. 2005; Lee *et al*. 2009; Palanichamy *et al*. 2009).

Although B cells from bone marrow exhibited high percentages of $CD24^{hi}CD38^{hi}$ cells, they still remained immature and unable to leave bone marrow in

normal conditions, therefore a direct participation in kidney or systemic tolerance is unlikely. Furthermore, bone marrow sampling is unpleasant, expensive, requires certain surgical elements and it was not ethically justifiable to obtain B cells from kidney transplant recipients with stable function.

The presence of Transitional B cells was evaluated in peripheral blood, lymph nodes and spleen from healthy volunteers and cadaveric donors to establish which tissue exhibited the highest percentages of Transitional B cell. Results demonstrated that the highest percentages of Transitional B cells were found in peripheral blood. Cuss and colleagues evaluated $CD19^{+}CD24^{hi}CD38^{hi}$ cells in spleen and lymph nodes and like the results obtained in this thesis, both tissues exhibited a low presence of Transitional B cells. On the other hand, they found high percentages of Transitional B cells in cord blood, but not in peripheral blood (Cuss *et al*. 2006; Lee *et al*. 2009). Of note, their average percentage was 2.6% of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in peripheral blood samples, whereas this study found a range between 0.5-15% of $CD20^{+}CD19^{+}CD27^{-}CD24^{hi}IgM^{+}CigD^{+}CD38^{hi}$ Transitional B cells in healthy control and patient samples. The differences found can be due to the different gating strategies used to identify all B cells subsets, especially Transitional B cells.

Peripheral blood was the best source to evaluate Transitional B cells in kidney transplant recipients, not only because of the higher percentages exhibited compared to other tissues, but also because it was the least invasive source from which to obtain samples.

4.4.4 Tolerant recipients exhibited higher percentages of Transitional B cells than non-tolerant patients

B cell subsets, especially Transitional B cells, were measured in peripheral blood samples from healthy controls and kidney transplant patients. Tolerant patients exhibited the highest percentages of Transitional B cell compared to other patient groups.

B cells and kidney transplantation tolerance was linked for the first time in 2010 by Brouard's group (Brouard *et al*. 2007; Pallier *et al*. 2010). They defined drug-free long-term graft function (DF) patients as patients with long-term acceptance of mismatched kidney allografts, after immunosuppressive drug withdrawal, following

kidney transplantation. They found that tolerant patients displayed a high number of peripheral B cells, particularly Memory B cells, and that these B cells exhibited an inhibitory and survival profile.

During the same year, two large-scale studies focused on identifying a signature of tolerance in kidney transplant patients, one from Europe led by Robert Lechler (Sagoo *et al*. 2010) and one from the USA led by Vicki L. Seyfert-Margolis (Newell *et al*. 2010), were published together in the Journal of Clinical Investigation. Both studies revealed an expansion of peripheral blood B cells, mainly the Naïve and Transitional population, and an increased expression of multiple B cell-related genes.

One year later, Cohelo's group identified a rare group of kidney-transplanted individuals, defined as operational tolerance (OT), able to maintain normal graft function with an immunocompetent immune system after the complete withdrawal of immunosuppressive drugs. They found that tolerant patients displayed preserved numbers of B cells, Naïve B cells and Bregs (defined as $CD19^{+}CD24^{hi}CD38^{hi}$). Tolerant patients also displayed preserved BCR diversity, and a preserved CD40/STAT3 signalling pathway in Bregs (Silva *et al*. 2012).

Data from the study presented in this thesis was obtained from a selected group of patients chosen from the GAMBIT study. 12 newly identified tolerant recipients and three tolerant recipients included in the IOT study were recruited for the GAMBIT study and used in this research project. After three years of B cell subset phenotypic analysis, only partial confirmation of what was described by the previous studies could be made. Even when results from this thesis agreed with other studies regarding the expansion of B cells (in particular Transitional B cells) in tolerant recipients, results regarding the Memory or Naïve populations differed from the findings in previous studies. Discrepancies in the results can be explained because previous groups evaluated numbers of B cells rather than percentages.

In conclusion, the number and percentages of Transitional B cells were different in tolerant recipients compared to patients maintained with immunosuppressive therapy, however, no difference was observed between tolerant recipients and healthy control. These results opened several questions regarding why Transitional B cells were maintained in tolerant patient but not in other patient groups, what was the immunological effect of the cells in graft function and whether the immunosuppressive

drugs had an effect on Transitional B cell survival.

4.4.5 The effect of immunosuppressive drugs in B cell viability *in vitro*

Because Transitional B cells percentages between tolerant and healthy control samples did not exhibit any significant difference, a possible detrimental effect of immunosuppressive drugs was evaluated in B cell survival *in vitro*.

The detrimental effect of immunosuppressive drugs in B cell survival was measured as apoptosis induction and cell death. The expression of BAFFr was linked to apoptosis induction. The main reason for doing this is that this particular receptor and its ligands have a crucial role in survival signalling and maintenance of mature B cells (Craxton *et al*. 2005; Li *et al*. 2012). Furthermore, defects in BAFFr expression impairs B cell development and prevents the formation of follicular and marginal zone B cells (Rauch *et al*. 2009).

Results from this thesis showed that down-regulation of BAFFr was a specific indicator of apoptosis in the B cells, for this reason, the expression of this receptor was measured on B cells from kidney transplant recipient. Stable and chronic rejector recipients exhibited a lower expression of BAFFr compared to healthy controls, but tolerant recipients did not exhibit any difference compared to healthy volunteers; this indicated that B cells from stable and chronic rejector were more sensitive than B cells from tolerant patients. Pallier *et al*. reported that drug-free long-term graft function (DF) patients exhibited a transcriptional profile favouring B cell survival. These patients displayed an increased BAFFr/BAFF ratio compared to patients with stable graft function, healthy individuals, and patients with chronic rejection (Pallier *et al*. 2010). This study's findings matched those of Pallier *et al.*, showing differential survival signals between tolerant patients and stable and rejector patients, but unlike Pallier *et al*., no differences between tolerant patients and healthy individuals were found.

BAFFr expression in stable and chronic rejector was lower than in B cells from healthy control. A possible reason for this could have been the toxic effect of immunosuppressive drugs in B cell viability. The effect of tacrolimus, MMF and steroids was evaluated, but no clear pattern was found that could explain or associate these drug levels with B cell death.

Literature reported different effects of these three drugs, for example, Beattly and colleagues evaluated the effect of tacrolimus on EBV-infected B cells and found that tacrolimus promoted EBV-infected B cells growth through protection from cell death. In addition, they demonstrated that tacrolimus inhibited Fas-mediated apoptosis (Beatty *et al*. 1998). This report agrees with the results shown before, as no difference was observed in B cells cultured with tacrolimus.

Another example is the Ganschow study, which demonstrated for the first time that using the immunosuppressive drug MMF resulted in MMF-induced B-cell dysfunction and depletion in clinical transplantation. They presented the case of a 9-yr-old girl with primary hyperoxaluria type 1 and systemic oxalosis. The post-operative immunosuppression consisted of cyclosporine, prednisolone, and MMF. Four weeks post-transplant, serum IgG concentration and B cell count were critically low but this effect was reversed when MMF was stopped (Ganschow *et al*. 2001). In this thesis, results *in vitro* did not demonstrate any difference in viability when MMF was added in B cell cultures, but interaction with other drugs *in vivo* could cause the detrimental effect observed by this group.

Finally, Lill-Elghani's group found in humans that glucocorticoid induced apoptosis of $CD10^{+}CD19^{+}$ early B cells in the bone marrow, whereas IgD^{+} mature B exhibited glucocorticoid resistance (Lill-Elghanian *et al*. 2002). Studies of B cell subsets in lung transplant recipients treated with steroids, tacrolimus, and MMF showed a lack of differences in the absolute number of B cell subsets within the first year of follow-up (San Segundo *et al*. 2012). Kathryn Wood's group demonstrated that in B cell repopulation after Alemtuzumab induction, a transient increase of Transitional and Naïve B cells could be seen, and this was not affected by treatment with immunosuppressive therapy consisting of tacrolimus, MMF and steroids (Heidt *et al*. 2012). In this thesis, B cell viability and apoptosis was severely affected by steroids, but again these tests were performed only *in vitro*, therefore no real drug effect can be concluded using this method.

4.4.6 CD20 and CD19 protein expression in B cells subsets

CD20 and CD19 molecules are B cell markers that allowed the identification of the B cell population within PBMCs. CD19 is one of the earliest cell surface molecules

related to B cell lineage differentiation and has been shown to provide a co-stimulatory signal for activation through the BCR (Horvath *et al*. 1998). CD20 is another early B cell differentiation antigen identified and CD20-specific monoclonal antibodies are commonly used for the treatment of B cell malignancies and autoantibody-mediated autoimmune diseases. Despite that, the role of CD20 in human B cell physiology remains elusive (Kuijpers *et al*. 2010).

The CD20-codifing gene, *MS4A1*, was one of the genes found in the IOT study (Sagoo *et al*. 2010) to be differentially expressed in tolerant recipients compared to other patients groups. This gene was also found up-regulated in tolerant patients from the ITN study (Newell *et al*. 2010) and in the study from Brouard *et al* (Brouard *et al*. 2007). *MS4A1* was the only common gene found in these studies. Moreover, only the CD20 transcript from urine sediments was significantly higher in tolerant patients compared to patients with stable function and healthy controls (Newell *et al*. 2010).

Transitional B cells express significantly higher levels of CD20 compared to Naïve and Memory B cells (Cuss *et al*. 2006). This was confirmed by the current study and it was also found that CD20 density in cell membrane was higher in Transitional B cells compared to other B cell subsets. Despite CD20 being higher in tolerant recipients, no suggestion of a direct relationship between CD20 and tolerance can be made, due to the lack of knowledge of CD20 function in the B cell population. The only evidence of a human case without CD20, demonstrated that CD20 has a central role in the generation of T cell-independent (TI) antibody responses, but not in B cell proliferation, activation or survival (Kuijpers *et al*. 2010).

Unlike *MS4A1,* CD19 encoded-gene, *CD19*, was found in position 489 in the IOT study (Sagoo *et al*. 2010); this is interesting because even when both markers are fully expressed on B cells, only CD20 was increased in tolerant recipient. This result opens a new approach about the potential unknown role of CD20 in transplantation tolerance.

The clinical use of Rituximab, a chimeric monoclonal antibody against CD20 used to deplete B cells in haematological (Maloney *et al*. 1997) and autoimmune (Virgolini *et al*. 2004) diseases, and more recently in transplantation (Salama *et al*. 2006), was another important aspect to consider. Because Transitional B cells express the highest levels of CD20, these cells are probably the most sensitive population to be

affected by Rituximab. This suggests that Rituximab is not only destroying harmful predecessors of antibody-producing cells, it is also destroying, (with high efficiency) B cells with immunoregulatory properties.

Studies from Alausa and colleagues presented the case of a 49 years old Caucasian male with early acute kidney allograft rejection that was refractory to high doses of steroids and thymoglobulin. This patient was treated with Rituximab and Muromonab (anti-CD3ε) and after 9 months post-transplantation, he remained rejection free with a serum creatinine of 1.7 mg/dL (Alausa *et al*. 2005), suggesting that Rituximab in combination with an anti-CD3ε delayed graft rejection.

Faguer and colleagues did a pilot study in eight renal-transplant patients presenting acute humoral rejection. They assessed the efficacy of Rituximab (375 mg/m^2 weekly) for 3 to 5 consecutive weeks, in addition to plasma exchange, steroids, MMF, and tacrolimus. After a follow-up of 10 months (range 7–23), patient and graft survival was 100% and 75%, respectively. Renal function improved in six cases (levels of creatinine was decreased), graft loss occurred in two cases, and four patients had infectious complications. At the final follow-up, DSA had disappeared or decreased in four cases. They concluded that Rituximab therapy, in addition to plasma exchange, might be beneficial in renal transplant patients presenting acute humoral rejection (Faguer *et al*. 2007).

On the other hand, Tyden and colleagues reported a 3-years study of ABO-incompatible renal transplant patients in which forty-four received Rituximab and forty-seven received placebo. They concluded that a tendency was seen toward fewer and milder rejections during the first 6 months in the Rituximab group, but after the 3-year follow-up, they did not find a significant difference in the development of donor-specific antibodies. There was also no difference in the number of graft losses between the groups, but there was a statistically significant difference in the mortality, as eight deaths were found in the Rituximab study group *versus* none in the placebo group (Tyden *et al*. 2012).

Clatworthy *et al*. planned to recruit hundred and twenty patients in their clinical trial, comparing Rituximab *versus* Daclizumab (anti-CD25 monoclonal antibody) as induction therapy in patients undergoing renal transplantation, but the study was

suspended after recruitment of the first thirteen patients, owing to an excessive incidence rate of acute cellular rejection in the Rituximab group. Patients who received Rituximab had a rate of acute rejection that was not only higher than the rate in the control group (83% vs. 14%), but also higher than that previously observed among patients who had not received induction therapy (35%) (Clatworthy *et al*. 2009), suggesting that Rituximab was an unsuccessful induction therapy.

Differences and contradictions of Rituximab clinical trials in transplantation, such as the ones mentioned above, do not clarify the effectiveness of Rituximab in improving graft survival or reducing acute or chronic rejection. In turn, the B cell role in renal transplantation is also not clarified, and the understanding of a potential role of regulatory B cell in graft survival is fundamental to address this question.

4.4.7 B cells from kidney transplant patients up-regulated CD25 and CD86 expression after cognate CD40 activation

After the phenotypic characterisation, a functional characterisation was performed in B cells from healthy controls and patients, measuring up-regulation of activation molecules and cytokine production after CD40 and CpG activation.

Up-regulation of activation molecules is an important indicator of cell activation. This parameter was measured by comparing non-simulated with stimulated B cells in PBMCs samples from healthy volunteers and kidney transplant patients.

No differences in CD86 expression were found between healthy controls and patient samples in this study; B cells from all patient groups up-regulated CD86 after CD40L activation. Pallier *et al*. found that total non-activated B cells of tolerant patients displayed significantly higher levels of CD80, CD86, CD40, and CD62L compared to other transplant patients. They proposed that B cells from tolerant patients expressed an activated-memory phenotype with a positive expression of co-stimulatory molecules, but when B cells from patients were activated for different lengths of time using different stimuli (CD40L, CD40L+anti-IgM, CD40L+CpG), no significant difference between activation molecules was observed (Pallier *et al*. 2010). Newell *et al*. also found that non-activated $CD86^{+}CD19^{+}$ B cells were significantly higher in samples from tolerant patients compared to healthy controls (Newell *et al*. 2010), but because all percentages were no higher than 5% of positive cells, it does not seem plausible to argue

that these cells were generally activated.

Although no differences were observed in CD86 up-regulation, the CD25 molecule was significantly increased in B cells from tolerant recipients. When the expression of this molecule was studied in B cell subsets, $CD27^-$ (Naïve and Transitional) B cells were the main population responsible for the differential expression. The high expression of CD25 in human IL-10-competent B cells was observed by Tedder *et al*. (Iwata *et al*. 2011). However, Pallier *et al*. did not find any significant differences of CD25 expression in activated B cells (CD40L, CD40L+anti-IgM, CD40L+CpG) from patients or healthy controls samples (Pallier *et al*. 2010). Different findings from the latter study might be explained by the use of an anti-CD40 antibody, while in the study presented here, CD40L-transfected cells were used for B cell activation since activation with transfected cells was always higher compared to the soluble antibody (data not shown). Tedder *et al*. tested a recombinant CD40L and a soluble anti-CD40 monoclonal antibody, and they observed 3.2% and 1% of $IL\text{-}10^+$ CD19 cells after activation, respectively. They used recombinant CD40L in combination with LPS or CpG, obtaining percentages of 12.1% and 9.3% of $IL\text{-}10^+$ CD19 cells, respectively, suggesting that the recombinant CD40L and a soluble anti-CD40 monoclonal antibody were not enough stimuli for IL-10 production in their study (Iwata *et al*. 2011).

In conclusion, results from this thesis suggested that the expression of CD25 by B cells is important in tolerance. CD25 is part of the IL-2 receptor, and IL-2 induces survival signals in B cells. Since B cell survival was decreased in stable and chronic rejector but not in tolerant recipients, and high expression of CD25 was observed only in $CD27^-$ B cells from tolerant recipients after CD40L activation, it is suggested that high expression of the IL-2 receptor by B cells may indicate better B cell viability. Since $CD27^-$ B cells from tolerant recipients exhibited better viability and endure better CD40 activation, these cells may produce more IL-10 than B cells from stable and chronic rejector, contributing thereby in the tolerant state.

4.4.8 B cells from kidney transplant patients up-regulate TLR-9 after CpG activation

TLR-9 expression was measured on B cells by intracellular staining to ensure

that these cells were responding to CpG activation. This staining was difficult to standardise as TLR-9 is not express on the cell surface after activation. In spite of this, using a modified intracellular staining method and a second blocking step, the staining of TLR-9 expression was detected; unfortunately these results could not be compared with published data as gene expression, and not protein expression, has been used to measure TLR-9 expression.

Liang *et al*., using RT-PCR, described that human B cells from patients with chronic lymphocytic leukaemia expressed high levels of TLR-9 when cells were CpG ODNs activated. They also found that CpG ODN induced IL-10 production, which in turn, induced STAT-1 phosphorylation and B cell apoptosis (Liang *et al*. 2010).

In this thesis, IL-10 production and TLR-9 expression after CpG activation were measured in B cells within PBMCs and a correlation between both parameters was observed (data now shown), demonstrating that IL-10 production and TLR-9 expression were both induced by CpG activation.

4.4.9 Transitional B cells secrete IL-10 after CD40 activation, and Memory B cells secrete IL-10 after CpG activation

Transitional B cells, Regulatory B cells or IL-10-competent B cells are different definitions for IL-10-producing B cells. The importance of IL-10 production lies in the role of IL-10 as an anti-inflammatory cytokine in the immune system. The next question that this thesis addressed was whether this cytokine was differentially secreted by B cells from tolerant patients.

In humans Duddy *et al*. reported that B cells secreted IL-10 after CD40 stimulation. Interestingly, when CD40 stimulation was performed together with BCR activation, instead of IL-10, B cells produced more TNF-α, LT, and IL-6 (Duddy *et al*. 2004). Further investigation by Tedder's group revealed that CD40L activation induced IL-10^+ B cells, but dual stimulation of CD40 and CpG for 48 hours induced even higher frequencies and numbers of IL-10^+ B cells. The phenotype of these IL-10-competent B cells was similar to the phenotype exhibited by Transitional B cells (Iwata *et al*. 2011). In summary, CD40 and CpG activations were the main stimuli to obtain IL-10 from B cells, whereas CD40 targeted Transitional (Blair *et al*. 2010) and Naïve cells (Iwata *et al*. 2011), and CpG induced IL-10 secretion from all B cells (Liang *et al*. 2010).

Intracellular staining was the first technique used to evaluate IL-10 production by B cell subsets after CD40L and CpG activation, but after several attempts it was discovered that it was impossible to identify Transitional B cells because their specific markers were down-regulated after three days of culture plus PMA activation. IL-10 production in sorted B cell subsets from healthy individuals was evaluated to understand which activation was stimulating which B cell subset, for further extrapolation of these data into patient samples results. Results demonstrated that Transitional B cells were the main population responding to CD40-CD40L interaction, which concurred with published data, but it was also found that predominantly Memory B cells responded to CpG activation.

From the results obtained from the sorted B cell subsets, CD40L and CpG were used separately in patient samples in order to measure IL-10 secretion produced by Transitional B cells, as this population was responding specifically to CD40L and was the main population increased in tolerant patients. The initial idea was to use CpG as a positive control for IL-10 secretion and expression, but based on the results that were obtained, CpG activation was used to evaluate possible immune-regulatory mechanisms related to Memory B cells *via* IL-10 production.

4.4.10 B cells from tolerant recipients produced higher levels of IL-10 and lower levels of TNF-α than B cells from chronic rejector

After all the required standardisations, IL-10 was measured in patient samples; a significant increase of IL-10-producing B cells was found in tolerant patients compared to chronic rejector, after CD40 or CpG activation. This means that both Transitional and Memory B cells from tolerant recipients were producing IL-10 after activation.

The results on IL-10 production observed in patient samples showed several discrepancies with published data. For instance, Pallier *et al*., measured cytokine secretion in the supernatants of B cell non-stimulated or stimulated with CD40L, CD40L/CpG, or CD40L/anti-IgM after 40 hours of activation, but they did not observe any difference in IL-10, IL-6, and TNF-α levels between the different groups of patients (Pallier *et al*. 2010). In this thesis, early attempts to activate B cells using soluble CD40 antibody were invariably unsuccessful. It seems that in Pallier's study they were not

activating B cells either, as they could not find evidence up-regulation of activation molecule after their different activation methods.

Newell's data reported a statistically significant increase in IL-10^{+}CD38^{+}CD24^{+} Transitional B cells in the tolerant group relative to the stable or healthy control, after activation with PMA and ionomycin. Although the differences measured were statistically significant, they recognised that the overall percentages were extremely low, with many samples lacking any IL-10–producing cells, and with a large overlap of IL-10 expression between groups. They suggested that this could be caused by the stimulation conditions that were limited in length and intensity in order to maintain the phenotype of the subsets. In this thesis, PMA and ionomycin did not specifically increase IL-10 production in total B cells, and high intensity stimulation down-regulated CD24 and CD38. Unlike the current study, Newell's study did not confirm the intracellular data measuring protein levels from the supernatants.

Sagoo *et al*. also evaluated production of TGF-β, IL-10 and IFN-γ by intracellular staining, after *in vitro* stimulation of PBMC with PMA and ionomycin. Interestingly, using the same activation protocol than Newell's group, B cells from tolerant patients expressed higher percentages of CD20^{+}TGF-β^{+} B cells, but not IL-10^{+} or IFN-γ^{+} cells (Sagoo *et al*. 2010). While the IOT found higher percentages of CD20^{+}TGF-β^{+} B cells, the INT found higher percentages of CD20^{+}IL-10^{+} B cells in tolerant recipients after PMA and ionomycin activation. The discrepancies between those studies and the results in this thesis are possibly due to the differences in the length and kind of activation. As the aim of this study was to specifically target IL-10 pathway, activation lasted 72 hours, using CD40L cognate interaction and CpG.

Data presented in Coelho's study demonstrated IL-10 expression on Bregs after 48 hours of activation with mouse anti-human CD40 monoclonal antibody and soluble CD40L. CD19^{+}IL-10^{+} B cells were measured by intracellular cytokine staining. Interestingly, they did not measure IL-10 production in patient sample; data presented was obtained from only four healthy controls. In this thesis, IL-10 was measured not only by intracellular staining, but also in supernatants to confirm the validity of the results obtained by intracellular staining data in each kidney transplant recipient.

In terms of IFN-γ and TNF-α, unusual intracellular staining was observed in both cases. Staining of IFN-γ was strangely increased in B cells, but because ELISA did not exhibit any significant value, it was decided that the staining was due to changes in size scatter when B cells were activated. TNF-α was also difficult to measure in B cells from PBMCs samples; whereas differences in the levels of this cytokine were found between supernatants from activated and non-activated samples, the intracellular staining showed no differences between samples, showing all B cells (activated and non-activated) as positive for TNF-α. These results led to the conclusion that TNF-α was positive because of the effect of PMA and ionomycin rather than the activation with CD40L or CpG. For these reasons, only the data obtained from ELISA for this pro-inflammatory cytokine is presented in this thesis.

In conclusion, IL-10 was differentially secreted by B cells from tolerant patients compared to chronic rejector. In addition, B cells from chronic rejector secreted TNF-α instead of IL-10. This change in the secretion profile by B cells could be a consequence of the immunological responses exhibited in the different patient groups.

4.4.11 "Loss of Tolerance" kidney transplant recipients

The fact that some transplanted patients can obtain good graft function in the absence of immunosuppression is still puzzling. A number of questions regarding this clinical response are immediately raised such as how long does it last, can it be induced, is it stable overtime, and so on. In order to answer these questions, three patients that had lost their tolerant state were identified and studied to try to understand more this unusual phenomenon. This is the first time such group of patients has been evaluated and no previous records in the literature have been found. In patients from this group, the tolerant state was not permanent, but what cause the lost of graft function is still unknown.

In this study, it was found that although total B cell percentages remained without variation after losing tolerance, the percentages of Transitional B cells decreased over time. Loss of tolerance, accompanied by loss of Transitional B cells, supports the idea that Transitional B cells are participating in the tolerant state. Unfortunately, the quality of the samples did not allow for all the sets of experiments (to evaluate cytokine production or BCR activation) originally planned to be performed, but

when a comparison of IL-10 secretion between this group and tolerant recipients was made, it was observed that IL-10 secretion was absent in the “Loss Tolerance” group after CD40 and CpG activation.

These results further support a clear relation between tolerance and Transitional B cells as “Loss of Tolerance” patients have a decreased percentage of Transitional B cells and IL-10 secretion over time.

5 BCR signalling pathway and donor-specific response in kidney transplant patients

5.1 Introduction.

The immune response in kidney transplant recipients is commanded by the innate and the adaptive immune system (He *et al*. 2003). The innate response is a non-specific response, antigen independent, immediate, and exposure to antigen results in no immunological memory (Murphy *et al*. 2012). The adaptive response, on the other hand, is antigen-dependent and antigen-specific. There is a lag time between exposure and maximal response, and exposure to antigen results in immunologic memory (Murphy *et al*. 2012). Although both responses can participate or mediate rejection in kidney transplantation, the donor-specific response is still the most critical response involved.

The donor-specific response is principally initiated when gene products encoded in the MHC region from the donor cells are recognised as foreign by immune cells from the recipient (Halloran *et al*. 1986; Eremin *et al*. 2011; Murphy *et al*. 2012). Genes from the MHC region are highly polymorphic (Snell 1948) and were initially identified because of their presence in rejection of transplanted tissues (Snell 1951; Snell *et al*. 1951). There are two main classes of MHC molecules, Class I and II:

- Class I molecules are expressed in all nucleated cells. They are recognised by CD8 T cells, and three major loci, B, C and A, as well as other undefined minor loci are contained in their genetic complex (Sun *et al*. 1995).
- Class II molecules are only expressed on APCs such as DCs, macrophages and B cells. They are recognised by $CD4^+$ T cells; and the class II genetic complex also contains three loci, DP, DQ and DR (Bach *et al*. 1967; Mehra *et al*. 2003; Eremin *et al*. 2011).

Donor-MHC molecules can be identified by direct, indirect and semi-direct recognition. Direct recognition is when MHC molecules expressed on the surface of donor cells are directly recognised as foreign by recipient T cells (Warrens *et al*. 1994). Indirect recognition is when donor MHC proteins are recognised, internalised,

processed and presented as peptides by recipient APCs to recipient T cells, in a self-MHC restricted manner (Lechler *et al*. 1982). Finally, semi-direct recognition is when recipient APCs capture whole membrane blocks from donor cells, and immune cells from the recipient recognise donor MHC proteins contained in these membrane blocks through the direct pathway, together with indirect recognition of donor MHC antigens (Herrera *et al*. 2004).

The indirect pathway in kidney transplantation consists of the presentation of donor-antigenic peptides by self-MHC, in a self-restricted manner (Lechler *et al*. 1982; Shoskes *et al*. 1994). It has been associated with chronic rejection due to the long-term response triggered after donor-antigen recognition, internalisation and presentation by recipient APCs. B cells are also professional APCs because they present peptides in the context of the MHC class II. Unlike other APCs, B cells recognise donor-proteins with high specificity, due to the specificity exhibited by the BCR immunoglobulin to the foreign protein, therefore the recognition by the BCR is the first step in the indirect response induced by B cells (Lanzavecchia *et al*. 1985; Steele *et al*. 1996).

The B cell Receptor (BCR) is a transmembrane protein formed by both immunoglobulin and CD79 (Igα/Igβ). While the membrane-bound immunoglobulin is located in the outer surface of the cell and recognises foreign proteins, the heterodimer CD79 contains immunoreceptor-tyrosine-based activation motif (ITAM), in the intracellular tails, and they are responsible for the downstream signal activation (Murphy *et al*. 2012).

Activation of the BCR complex induces recruitment and activation of the protein tyrosine kinase Syk, which in turn, promotes BTK phosphorylation and PLCγ, Shc and Vav activation. Then, the BLNK adaptor protein continues the BCR activation downstream, resulting in the activation of the intermediate signalling protein Ras. Ras finally activates the MAP kinases, ERK, JNK and p38. These last proteins activate multiple signalling cascades, involving kinases, GTPases, and transcription factors that can change the cell metabolism, gene expression and cytoskeletal organisation. The complexity of BCR signalling allows triggering of different cell functions including survival, anergy, apoptosis, proliferation, and differentiation into antibody-producing cells or Memory B cells. This complexity deepens according to: maturation of the cell, the nature of the antigen, the magnitude and duration of BCR signalling, and signals

from other receptors such as CD40 or BAFFr. Therefore, the participation of B cells in the immune response is directly related to the different responses triggered after BCR activation (Sproul *et al*. 2000; Hasler *et al*. 2001).

B cells play a double role in donor-specific responses; through the BCR, they can recognise donor MHC molecules as antigens and then present them as donor-derived peptides in the context of MHC class II (Sproul *et al*. 2000), and they can differentiate into antibody-producing Plasma cells (Calame 2001; Calame *et al*. 2003). Evidence of clear participation of B cells in kidney transplant allorecognition is predominantly evident after the appearance of IgG donor-specific antibodies in the serum of the recipient. Presence of donor-specific antibodies indicates the existence of Memory B cells with BCRs (Ig) sharing the same specificities of the IgG donor-specific antibodies.

According to the third hypothesis in this thesis:

- Transitional B cells are able to modulate active allogeneic responses and therefore play a key role in inducing donor-specific tolerance.

Donor-specific response driven by B cells and their subsets, were evaluated in kidney transplant recipients. In order to answer this question, this chapter was divided in two aims:

Aim 1: Evaluation of the BCR signalling pathway, with a non-specific stimulus in patient samples and healthy controls.

Aim 2: Donor-specific antigen capture, internalisation and further antigen presentation by B cells to $CD4^+$ T cells. The idea was to measure BCR activation, donor-specific B cells, donor-proteins internalisation and the $CD4^+$ T cell response in patient samples, after antigen presentation by MHC class II expressing B cells.

5.2 BCR signalling pathway activation in kidney transplant patients.

5.2.1 BCR activation was quantified using ERK-p

The BCR signalling pathway was studied in healthy controls and patient samples after non-specific activation. Three proteins from the BCR signalling pathway were selected (BTK, BLNK and ERK) and phosphorylation of each protein was measured after activation by phospho-flow. BTK and BLNK were discarded from patient assays; the first protein because the BCR activation was not as optimal as expected, and the histogram did not show a well defined activation peak, and the second protein because it required specific buffers incompatible with those already used. Functional and technical complications, as well as the inconsistency of the staining made the measurement of these two proteins incompatible with the assay. ERK, on the other hand, showed a very stable staining with the highest separation between the negative and the positive control (Fig14 A).

A 6-colour phospho-panel was developed to measure the MFI of ERK-p in each B cell subset. Standardisation of this protocol was initiated by performing a time course to detect the maximum MFI difference between non-activated and activated samples. PMA was used as a positive control of ERK-p and 10 minutes was the minimum time required for maximum phosphorylation signal (Fig14 B). After 10 minutes, a plateau in the activation pattern was observed. The effect of CD40L as a co-activator of the BCR pathway was also measured, but no additive effect was found when both activations were performed together (Fig14 C). CD40L activation was then discarded from patient samples.

▲MFI was defined as the difference between the MFI of a 10-minutes stimulated sample with the MFI of a 0-minutes stimulated (or non-stimulated) sample (Fig14 D). A control sample was used to perform an inter-assay and intra-assay control. For the intra-assay control, three aliquots of the control sample were prepared and acquired at the same time; results in ▲ERK-p did not present significant differences (Fig14 E). For the inter-assay control, a control sample was acquired every time healthy control and patient samples were acquired (data not shown).

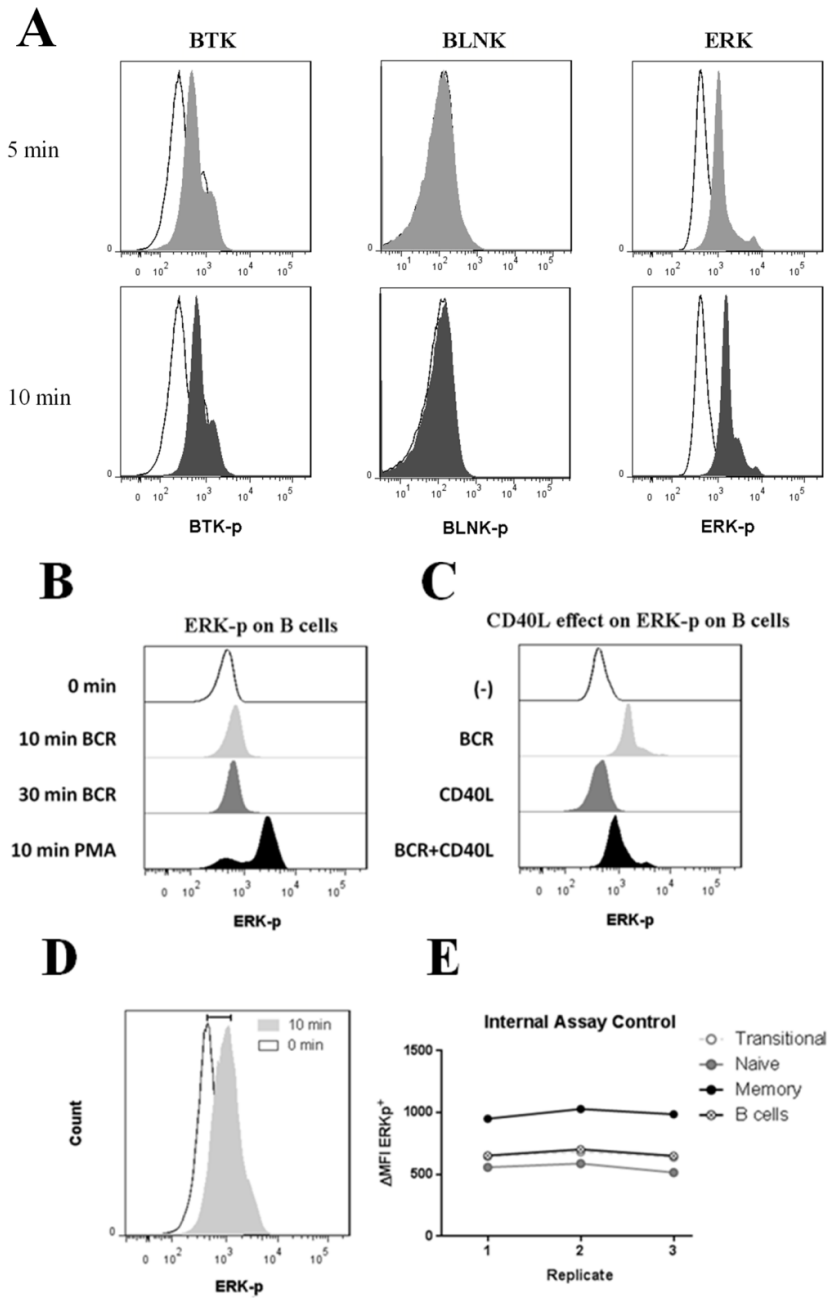


Figure 14: ERK phosphorylation.

BTK-p, BLNK-p and ERK-p were measured in 1.0x10^6 PBMCs from healthy volunteers after BCR-activation with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) during 0, 5 and 10 minutes at 37°C using phospho-flow. (A). A time course of ERK-p was measured in 1.0x10^6 PBMCs from healthy volunteers after BCR-activation with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) during 0, 10 and 30 minutes at 37°C using 0.1uM PMA 10 minutes as a positive control (B). The effect of CD40L as a co-stimulatory molecule was evaluated in ERK-p adding 0.5x10^5 CD40L-transfected L cells into 1.0x10^6 BCR-activated PBMCs (C). Difference in ▲ERK-p MFI between 1.0x10^6 non-activated PBMCs and 1.0x10^6 activated PBMCs with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) for 10 minutes at 37°C was displayed in a histogram (D). 1.0x10^6 PBMCs from the inter assay control were activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) during 0 and 10 minutes at 37°C;▲ERK-p MFI was measured in total B cells and B cell subsets (E).

5.2.2 ERK phosphorylation detection by phospho-panel in B cell subsets

BCR activation was determined by phosphorylation of the downstream protein ERK. Although this biological change allowed the development of an effective method to quantify BCR activation, the real challenge was to quantify this activation in each B cells subset. A 6-colour phospho-panel was designed to measure ERK-p in different B cell subsets after BCR activation.

The gating strategy began with the selection of the live population in PBMCs (Fig15 A). This population was very important to measure because the activation at 37°C, the intracellular staining protocol and the buffers used in the phospho-panel were very detrimental for B cell viability. The final protocol resulted in more than 80% of PBMCs viability. Then, from the live population, B cells were identified as $CD20^{+}$ cells (Fig15 B) and Memory B cells as $CD20^{+}CD27^{+}$ cells (Fig 15 C). Finally, from the $CD27^{-}$ population, Naïve and Transitional B cells were identified as $CD24^{+}CD38^{+}$ and $CD24^{hi}CD38^{hi}$, respectively (Fig15 D). Even with all the difficulties of the methodology used in the phospho-panel, the detection of each B cell subset was possible (with an acceptable separation and definition), in order to continue with the measurement of ERK-p. Delta ERK-p was then measured in total B cells, Memory, Naïve and Transitional B cells (Fig15 E).

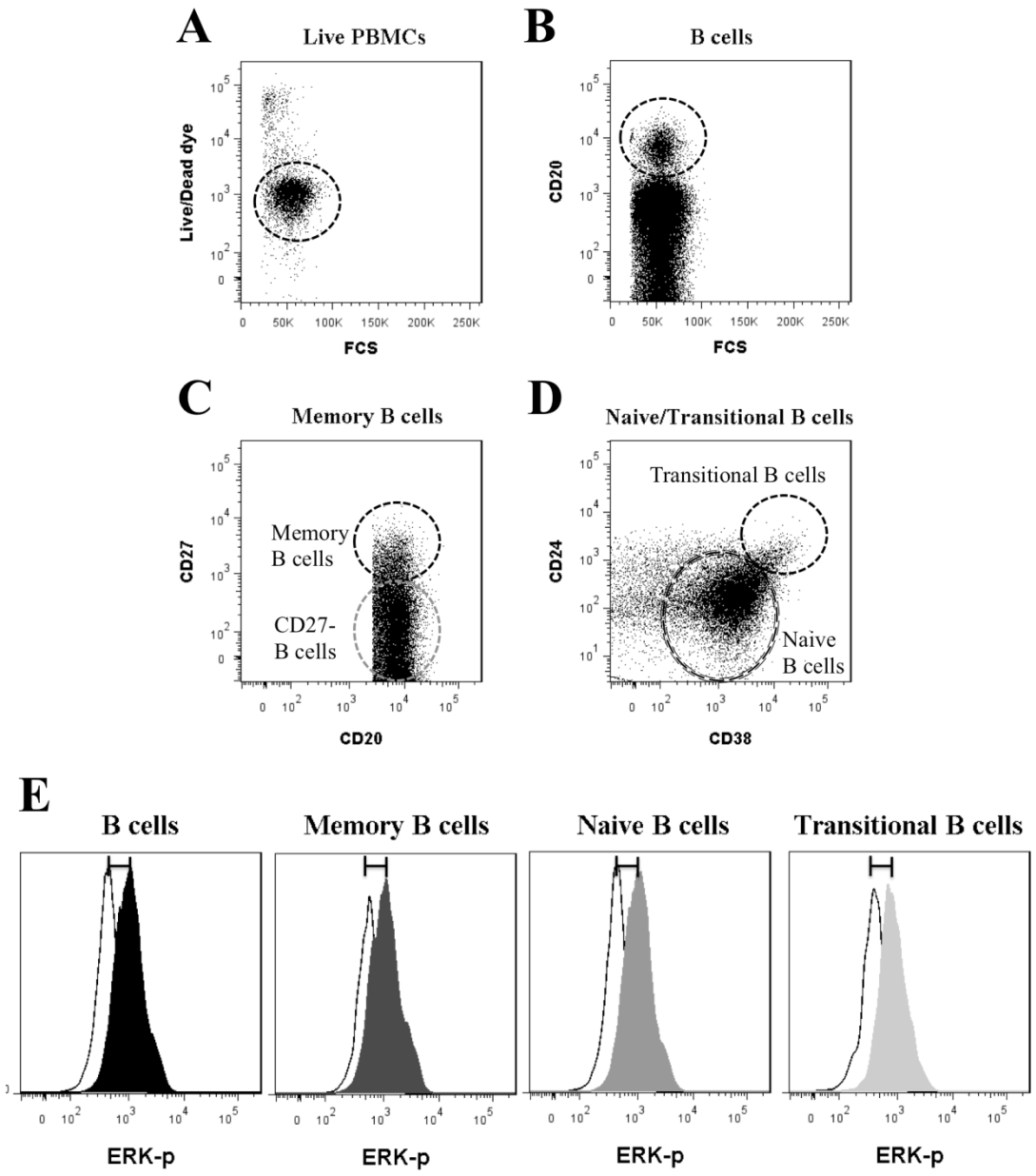


Figure 15: Phospho-panel gating strategy.

1.0x10^6 PBMCs were stained for 20 minutes at 37°C before activation with anti-IgM (20ug/ml)/anti-IgG (20ug/ml). Live cells were defined as Live/Dead$^-$ (A). B cells were defined as CD20$^+$ (B). Memory B cells were defined as CD27$^+$ (C) and from the CD27$^-$ population Naïve B cells were identified as CD24$^+$CD38$^+$ and Transitional were identified as CD24^{hi}CD38^{hi} (D). ▲ERK-p MFI was identified in total B cells, Memory, Naive and Transitional B cells as the difference between ERK-p MFI from non-activated samples (white histogram) and ERK-p MFI from samples activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) for 10 minutes at 37°C (colour histogram) using phospho-flow (E).

5.2.3 Distribution of IgM- and IgG-expressing cells in PBMCs from healthy control and kidney transplant patients

IgM percentages, and therefore IgG percentages indirectly, were measured in Memory and $CD27^-$ B cells from patient samples to ensure that the stimulus used in the BCR activation experiments were targeting Memory, Naïve and Transitional B cells (Agematsu *et al*. 1997; Agematsu *et al*. 1998).

The frequencies of IgM^+IgD^- (Fig16 A), IgM^+IgD^+ (Fig16 B), IgM^-IgD^- (Fig16 C) and IgM^-IgD^+ (Fig16 D) cells, within $CD27^-$ B cells, were measured by surface staining. At the same time, the frequency of IgM^+IgD^- (Fig16 E), IgM^+IgD^+ (Fig16 F), IgM^-IgD^- (Fig16 G) and IgM^-IgD^+ (Fig16 H) cells, within Memory B cells, were measured by surface staining in healthy controls and patient samples.

These tests found that $CD27^-$ cells were mainly IgD/IgM double positive (Fig16 B), while $CD27^+$ were divided in IgM^+IgD^+ B cells (un-switched class-Memory B cells) (Fig16 F) and IgM^-IgD^- B cells (switched class-Memory B cells) (Fig16 G).

Because of this mixture of IgM^+ with IgM^- cells, a combination of anti-IgM/ anti-IgG antibodies were used to target all B cell subsets after BCR activation.

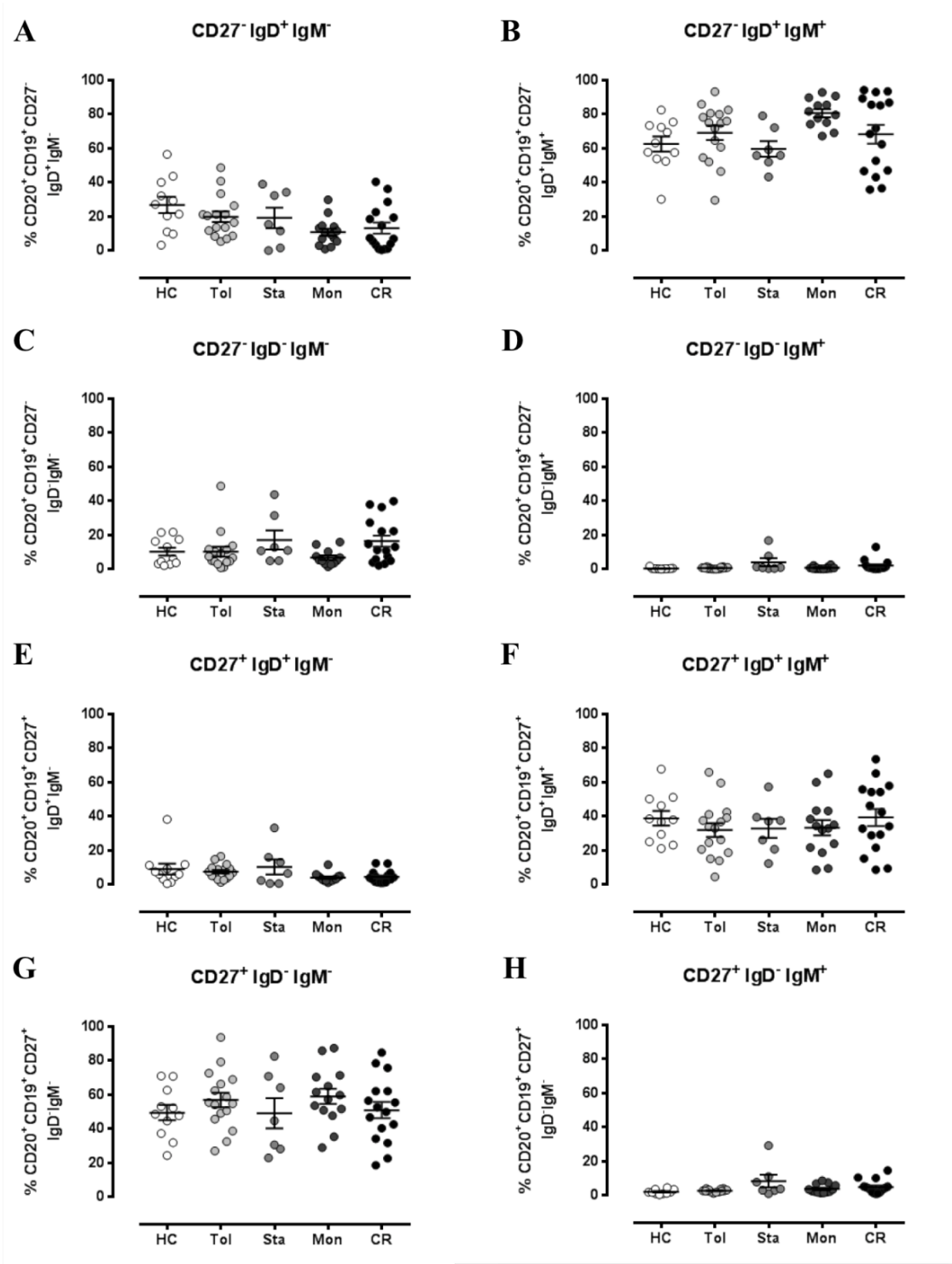


Figure 16: IgM/IgD distribution in CD27$^-$ and CD27$^+$ B cells from kidney transplant patients.

From the CD27$^-$ population (described in Fig1 B) IgM$^+$IgD$^-$ (A), IgM$^+$IgD$^+$ (B), IgM$^-$IgD$^-$ (C) and IgM$^-$ IgD$^+$ (D) populations were identified, and from the CD27$^+$ population (described in Figure 1 B) IgM$^+$IgD$^-$ (E), IgM$^+$IgD$^+$ (F), IgM$^-$IgD$^-$ (G) and IgM$^-$IgD$^+$ (H) populations were identified in PBMCs from healthy controls and patient samples using surface staining. Kruskal-Wallis test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant.

5.2.4 B cell subsets from tolerant recipients displayed defective ERK phosphorylation after BCR activation compared to healthy volunteers

PBMCs from healthy controls and patient samples were used to study the BCR activation in $CD20^+$ B cells and subsets. In order to activate all the B cell subsets at the same time, a combination of anti-IgM and anti-IgG activating antibodies were used. Previous tests had defined $CD27^-$ B cells as mainly IgM^+ cells (Fig16 B), while $CD27^+$ Memory B cells were both, IgM^+ (Fig16 F) and IgM^- (Fig416 G). Therefore, this dual stimulation allowed the activation of the BCR from Memory, Naïve and Transitional B cells simultaneously.

Results show that phosphorylation of ERK, after BCR activation, was reduced in $CD20^+$ cells from tolerant recipients (**p=0.0055) compared to $CD20^+$ cells from healthy control (Fig17 A). Regarding subsets, Memory B cells (Fig17 B) (**p=0.0049) and Naïve B cells (Fig17 C) (**p=0.0040) from healthy controls exhibited a higher BCR activation compared to samples from tolerant recipients only. Even when tolerant patients exhibited the same tendency of BCR activation in the Transitional population, the difference did not reach statistical significance (p=0.1277) (Fig17 D).

Using the same results, a different comparison between samples was performed; this time instead of comparing delta ERK-p between patient groups, delta ERK-p between B cell subsets from each patient group was compared. Within group comparison, healthy controls exhibited a higher activation in the Memory B cell subset compared to Transitional B cells (***p=0.0008) (Fig18 A). This tendency was lost in tolerant (Fig18 B), stable (Fig18 C) and monotherapy patients (Fig18 D), but was recovered in chronic rejector (*p=0.0361), in a lower scale (Fig18 E).

These results indicate that B cells and B cell subsets from tolerant recipients, exhibited a certain degree of anergy or unresponsiveness to general BCR activation, as delta ERK-p values were significantly different compared to healthy controls.

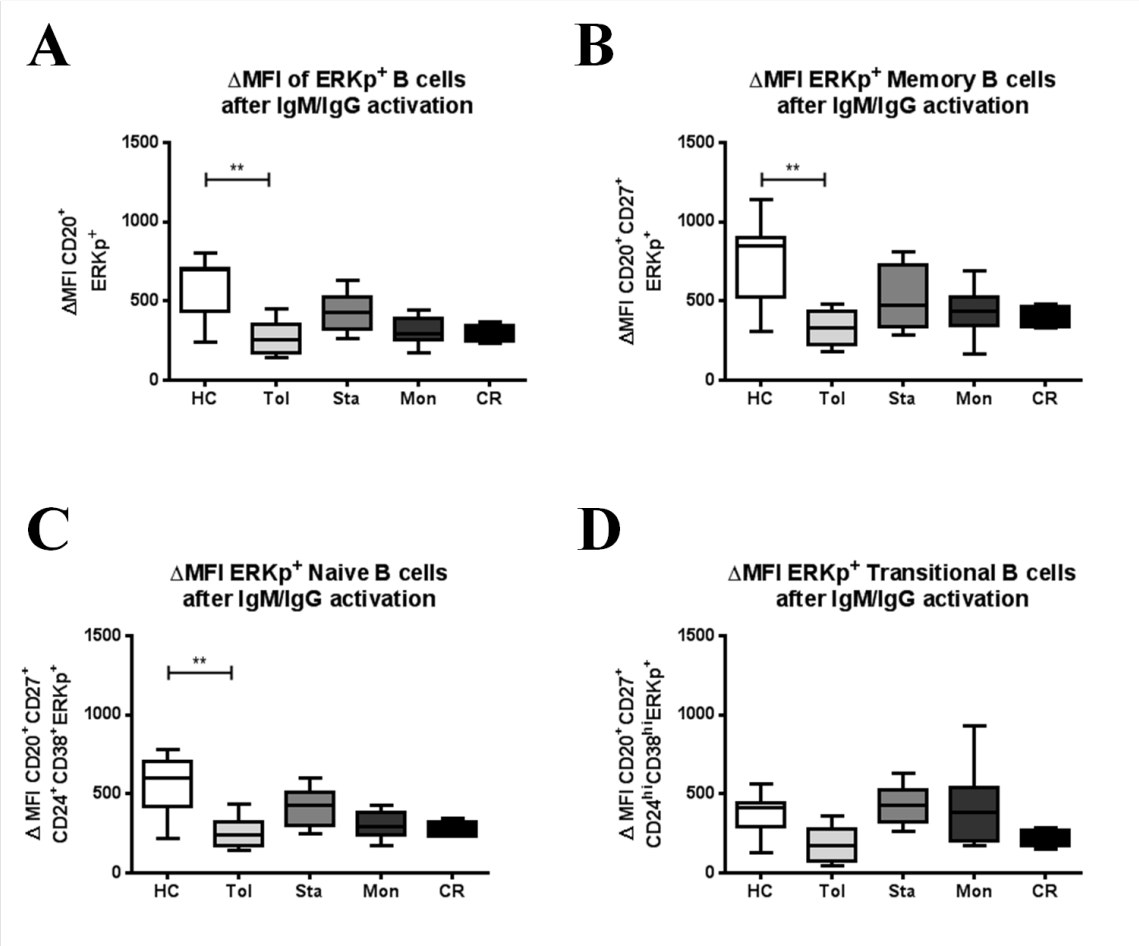


Figure 17: BCR activation measured in B cells subsets from kidney transplant patients.

PBMCs from control, healthy control and patient samples was thawed and rested in RPMI-1640 (Sigma) supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO_2 overnight. Next day, $3.0x10^6$ PBMCs from control, healthy control (HC), tolerant (Tol), stable (Sta), monotherapy (Mon) and chronic rejector (CR) were stained for 20 minutes at 37°C with the panel described in Figure 15, to identify ▲ERK-p in total B cells (A) Memory (B), Naïve (C) and Transitional (D) B cells. After surface staining, PBMCs were divided in three aliquots, and $1.0x10^6$ cells were activated with anti-IgM (20ug/ml)/anti-IgG (20ug/ml) or 0.1uM PMA (not shown) for 0 and 10 minutes at 37°C. ERK-p was identified using phospho-flow, and ▲ERK-p was calculated as the difference in ERK-p between non-activated samples and BCR-activated samples (described in Figure 14 D). Kruskal-Wallis test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant.

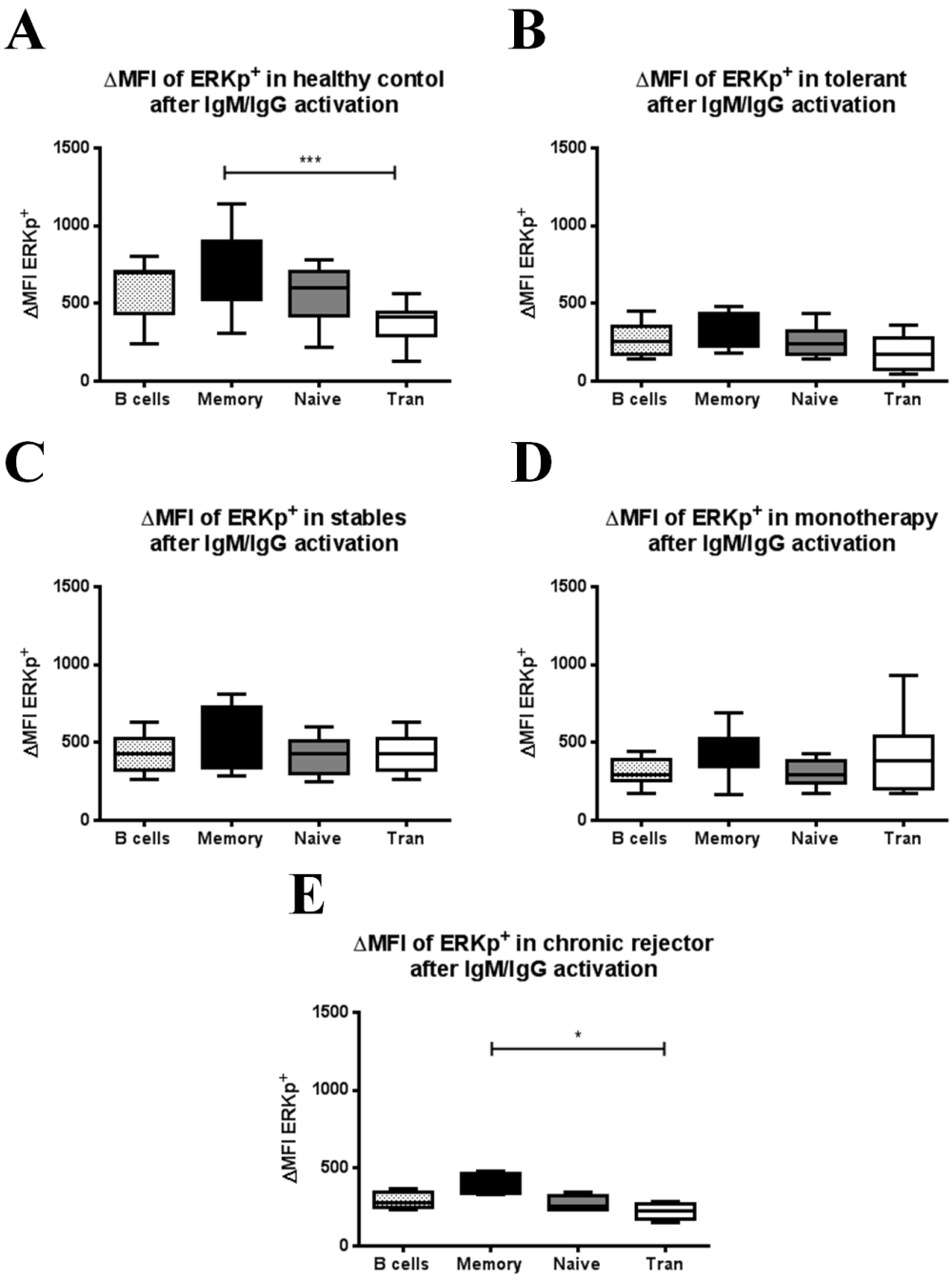


Figure 18: BCR activation measured between B cells subsets from kidney transplant patients.

▲ERK-p was compared between B cell subsets in PBMCs samples obtained from healthy control (A), tolerant (B), stable (C), monotherapy (D) and chronic rejector (E) using the same protocol described in Figure 17. Kruskal-Wallis test with a Dunn's multiple comparisons test was used, * $p<0.05$ was considered significant.

5.3 Donor-specific response in kidney transplant patients.

In terms of non-specific BCR activation, it was found that B cells from tolerant recipients exhibited a lower BCR activation compared to healthy volunteers. The next step was then to measure BCR activation and immune responses driven by B cells after specific activation, in this case with donor-specific antigens. The following section will describe the role of B cells as APCs for the alloresponse in different groups of kidney transplant patients.

5.3.1 Donor-proteins preparation

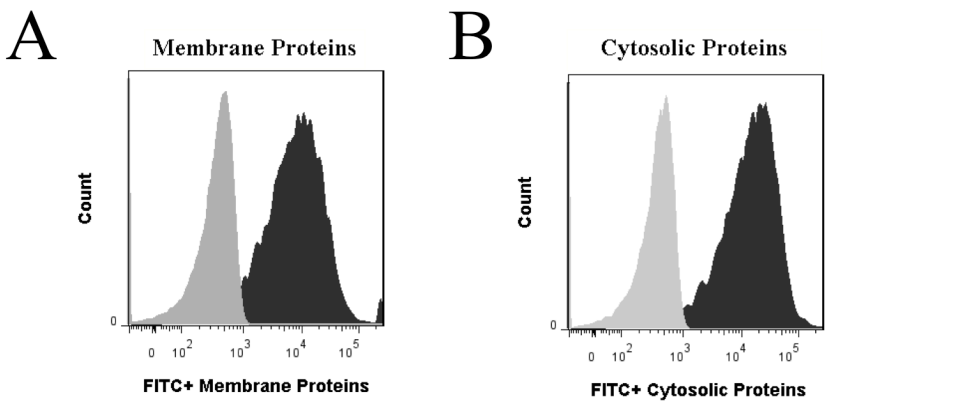
Donor-specific B cells can be identified by the ability of the BCR to bind donor-proteins; following on from this, an assay was developed to detect and measure donor-protein capture by recipient B cells. The challenge of this assay was to achieve protein fluorescent labelling, to then identify and locate these proteins, within recipient B cells.

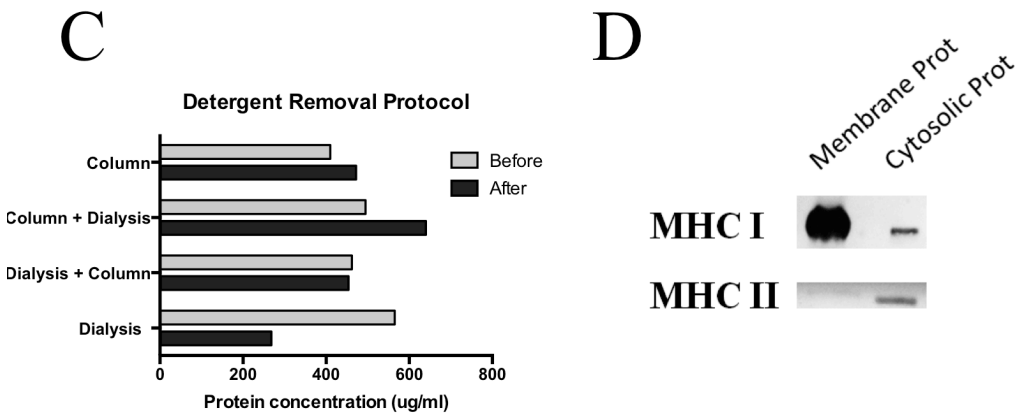
Protein preparation started with the enrichment of MHC class II^+ donor cells. For this reason, $CD2^+$ cells were removed from the PBMCs using positive selection with magnetic-beads. After the enrichment, donor cells were stained with CFSE, and proteins from the membrane and cytosol were isolated by centrifugation cycles with hydrophobic buffers. After the separation, histograms exhibited a $CFSE^+$ staining for donor-proteins obtained from the membrane fraction (Fig19 A) and from the cytosolic fraction (Fig19 B) of the cell. $CFSE^+$ protein samples ▬ were compared to unstained protein samples ▬ (Fig19 A&B).

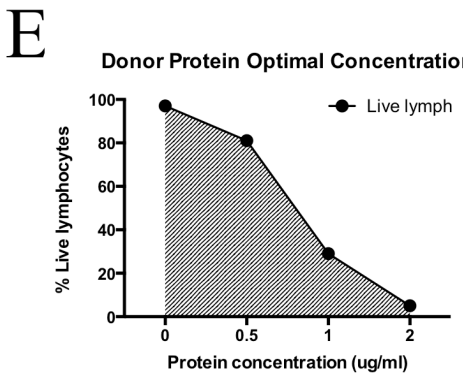
The optimisation of the solvent in which both fractions were contained was performed after protein isolation. While the cytosolic fraction was resuspended in a low-dose detergent solvent, the membrane fraction was resuspended in a high-dose detergent solvent. Cell toxicity is associated with high detergent concentrations; however, detergent can be removed using two methods; detergent-removal columns and dialysis. Detergent removal column protocols concentrated proteins in the samples, but also maintained high concentration of detergent in the protein preparations. On the other hand, dialysis halved the concentration of the proteins, but samples remained low levels of detergent (Fig19 C). Due to the toxicity produced by high detergent concentration, dialysis was used despite the fact that the concentration of the proteins was lower than the other method.

Once donor-proteins were in the appropriate solvent, MHC conformation was evaluated by western blot. MHC class I and II were found in both protein fractions (Fig19 D). Whereas higher concentrations of MHC class were found in the membrane fraction, MHC class II exhibited better definition in the cytosolic fraction.

Optimal protein concentration was established by measuring cell death from cultures at different donor-protein concentrations. More than 80% of live cells were observed when membrane protein concentration was between 0-0,5ug/ml (Fig19 E); therefore, this concentration range was used, as the viability was quite acceptable. Optimal culture conditions were also tested because the volume contained and the cell concentration strongly influenced the cell viability after protein addition. Then, 12 (Fig19 F) and 48 (Fig19 G) well-plates were used with 2.5, 5 and $10x10^6$ of PBMCs. Best culture conditions were found to be in 12 well-plate at $5x10^6$ PBMCs/4ml of media, with less than 0,5ug/ml of donor-proteins (Fig19 F).


A
Membrane Proteins
Count
FITC+ Membrane Proteins
B
Cytosolic Proteins
Count
FITC+ Cytosolic Proteins


C
Detergent Removal Protocol
Column
Column + Dialysis
Dialysis + Column
Dialysis
Before
After
Protein concentration (ug/ml)
D
Membrane Prot
Cytosolic Prot
MHC I
MHC II


E
Donor Protein Optimal Concentration
Live lymph
% Live lymphocytes
Protein concentration (ug/ml)

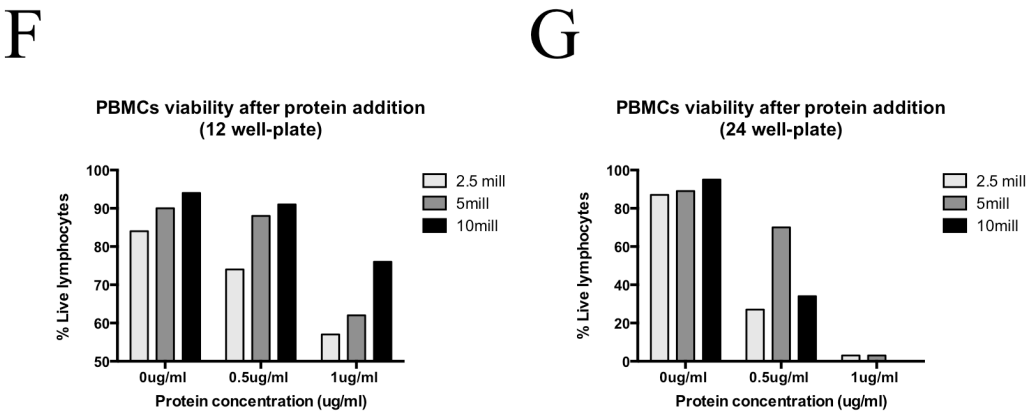

F
PBMCs viability after protein addition
(12 well-plate)
% Live lymphocytes
2.5 mill
5mill
10mill
0ug/ml
0.5ug/ml
1ug/ml
Protein concentration (ug/ml)
G
PBMCs viability after protein addition
(24 well-plate)
% Live lymphocytes
2.5 mill
5mill
10mill
0ug/ml
0.5ug/ml
1ug/ml
Protein concentration (ug/ml)

Figure 19: Donor-protein isolation.

Histograms were used to demonstrate the $FITC^+$ staining in unstained ▒ and $CFSE^+$ ■ protein samples from the membrane (A) and cytosolic (B) fraction of the cell after protein isolation. Four different methods were used to remove detergent from protein samples without drastically affecting protein concentration. Spin removal detergent columns, dialysis and combinations were tested to measure protein concentration after detergent removal (C). After isolation and dialysis of proteins preparations, western blot was performed to identify MHC class I and class II molecules using 20ug of protein per sample (D). Lymphocytes viability was measured after adding different concentrations of the $CFSE^+$ protein using a Live/Dead dye, and optimal viability was found when protein concentration was between 0 - 0,5ug/ml (E). Using the same technique, lymphocytes viability was measured after adding 0.5-1ug/ml of $CFSE^+$ proteins in 12 (4ml) (F) and 48 (2ml) (G) well-plate. Best condition was obtained when 0.5ug/ml of $CFSE^+$ proteins were cultured with $5.0x10^6$ PBMCS in a 12 well-plate with 4ml of complete media/well.

5.3.2 Donor-specific activation assays in two control samples

Before performing patient assays, the experiments were controlled with two control samples. The first control corresponded to a husband/wife pair sample in which the wife had become pregnant three times by her husband. It has been demonstrated that female transplant recipients who received an organ from their husband, with a single previous pregnancy, had a high graft survival rate, comparable to those with more than one pregnancy (Suzuki *et al*. 1997). It has been shown that a history of pregnancy induces high sensitisation in female recipients.

Based on this, proteins, prepared from MHC^+ class II cells from the husband, were tested with PBMCs from the wife. Autologous (wife) and allogeneic (husband) proteins were prepared and 0.5ug/ml of each preparation was added in PBMC cultures from the wife, to then measure the donor-protein specific B cells by flow cytometry. B cells were identified as $CD20^+$ cells from the live PBMC population cultured with autologous (Fig21 A) and allogeneic (Fig21 B) proteins; B cells were close to 3% in both samples. From the B cell population, $CFSE^+$ B cells that recognised autologous (Fig21 C) or allogeneic (Fig21 D) proteins were identified. Results revealed that incubation with allogeneic proteins exhibited a 0.416% of $CFSE^+$ cells compared to 0.035% of $CFSE^+$ cells from autologous proteins. The first control sample demonstrated specificity in the identification of donor-specific B cells despite the low number of events.

The second control pair sample was a kidney transplant recipient with known anti-HLA antibodies. “Negative’’ proteins were prepared from $CD2^-$ allogeneic PBMCs in which the allogeneic HLA proteins were not recognised by known recipient antibodies. “Positive” proteins were prepared from $CD2^-$ PBMCs that expressed HLA molecules recognised by known recipient antibodies.

B cells were identified as $CD20^+$ cells and monocytes as $CD14^+$ cells. Monocytes were used as a positive control for protein internalisation because these cells capture proteins by phagocytosis in a non-specific manner. B cells were cultured alone (Fig22 A), with “negative” (Fig22 B) or “positive” (Fig22 C) HLA proteins. From the same sample, $CD14^+$ cells were evaluated without proteins (Fig22 D), and with either the “negative” (Fig22 E) or the “positive” (Fig22 F) HLA protein preparations. A faint

but consistent difference was observed between the negative and the positive B cell samples (Fig22 B), but no difference was observed in the monocytes. Negative (Fig22 E) and positive (Fig22 F) samples within monocytes presented a similar percentage of protein capture.

In conclusion, the second control sample demonstrated specificity in the identification of donor-specific B cells, but it also demonstrated clear differences between two APCs exhibiting different internalisation processes. Whereas monocytes capture non-specific $CFSE^+$ proteins *via* phagocytosis, B cell internalise mainly donor-specific proteins, *via* BCR recognition.

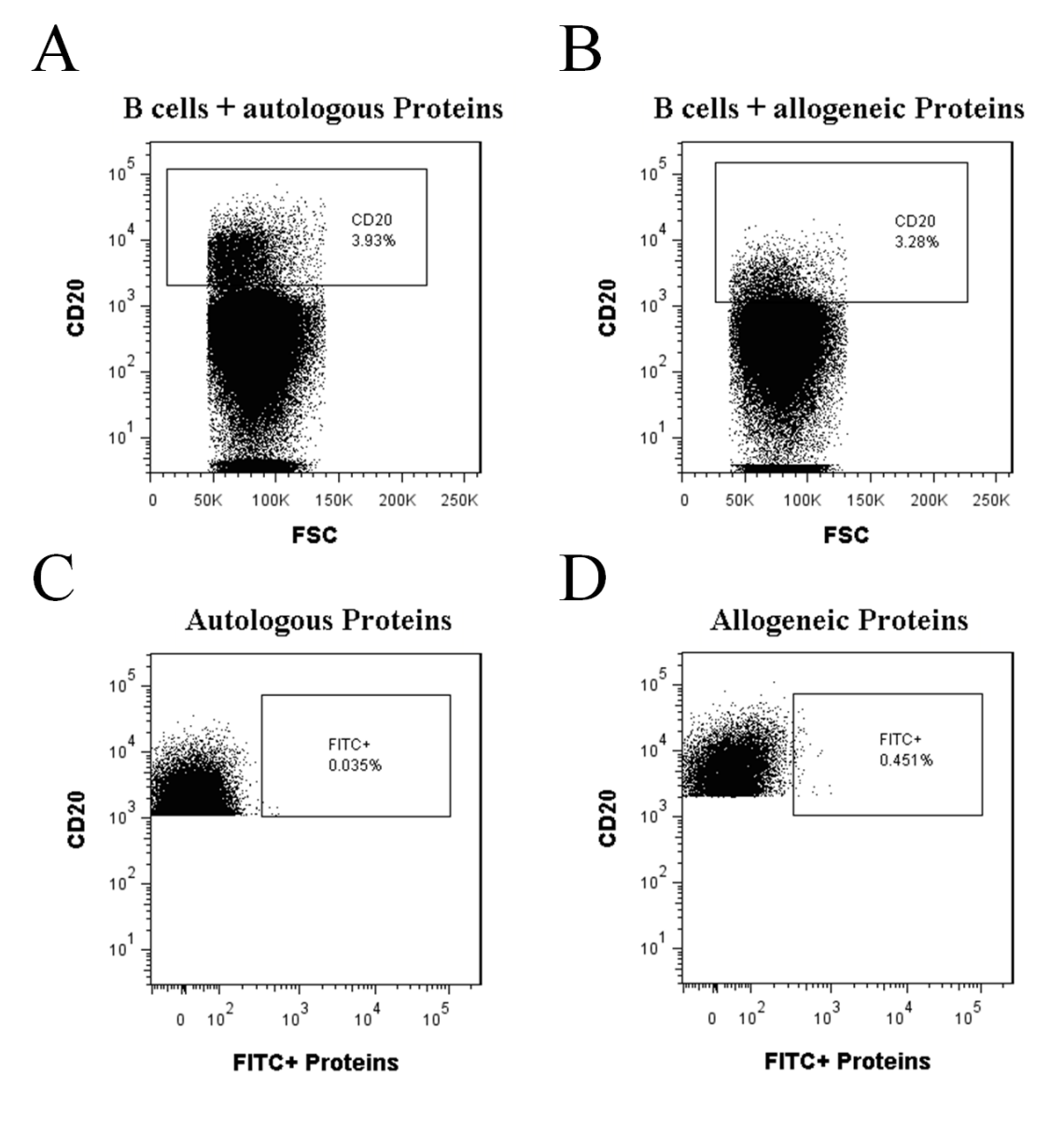


Figure 20: Control sample 1.

To perform the assays, 0.5ug/ml of autologous (A) and allogeneic (B) $CFSE^+$ proteins were cultured for 24 hours with $5.0x10^6$ PBMCS in a 12 well-plate with 4ml of complete media/well. Then, $CD20^+$ $FITC^+$ B cells were identified in samples with autologous (C) and allogeneic (D) $CFSE^+$ proteins using flow cytometry.

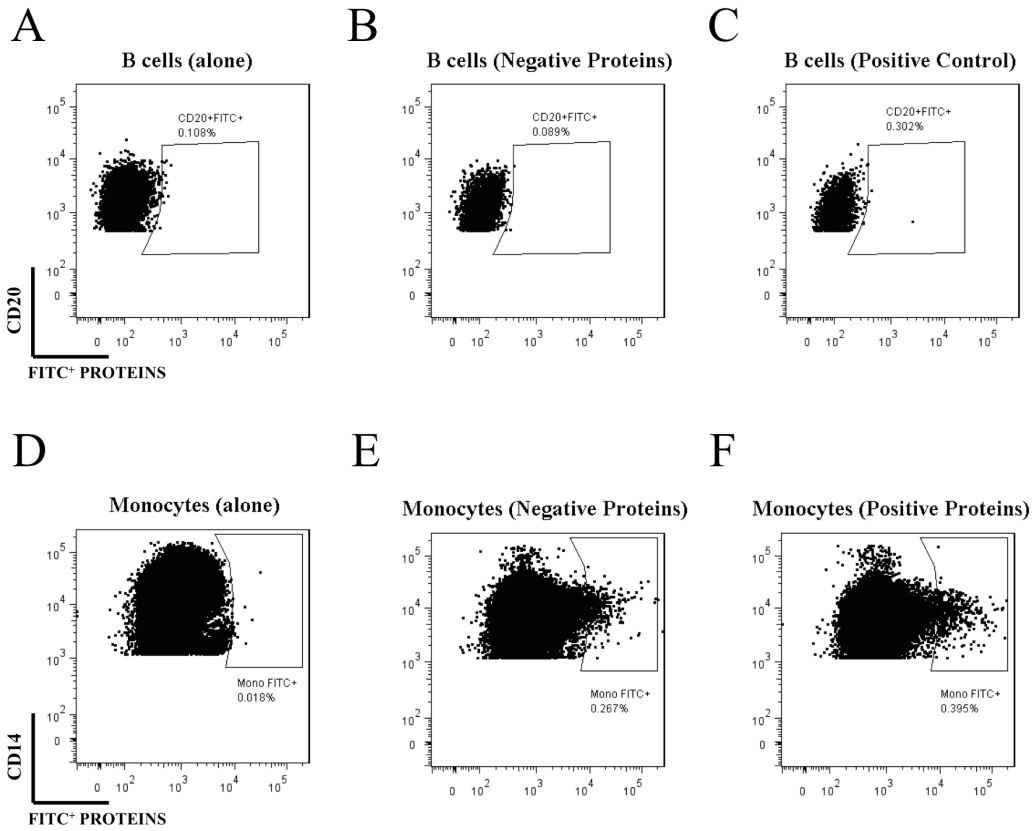


Figure 21: Control sample 2.

To perform the assays, $CFSE^+$ proteins were cultured for 24 hours with $5.0x10^6$ PBMCS in a 12 well-plate with 4ml of complete media/well. $FITC^+$ B cells were identified in samples with 0ug/ml (A), 0.5ug/ml of negative/autologous (B) and 0.5ug/ml of positive/allogeneic (C). $FITC^+$ CD14 monocytes were identified in samples with 0ug/ml (D), 0.5ug/ml of negative/autologous (E) and 0.5ug/ml of positive/allogeneic (F).

5.3.3 Donor-protein internalisation

After protein sample preparation, a time course was performed to define the minimum incubation time required to identify the donor-proteins inside recipient B cells. $CFSE^+$ cells were studied within monocytes and B cells from the control sample (described in 5.3.2) at 1, 2, 4, 8 and 24 hours after donor-protein addition. Both cells exhibited $CFSE^+$ cells after 24 hours (Fig20 A), hence the following assays were all performed after 24 hours of culture.

To confirm that CFSE staining found by flow cytometry corresponded to protein internalisation and not just a membrane bound protein, ImageStream images were used to identify the localisation of donor-proteins within the B cells, using the control samples (described in 5.3.2). Recipient PBMCs were cultured with $CFSE^+$ donor-proteins overnight. $CD20^+$ B cells were then sorted by staining with CD20 and MHC II markers for ImageStream acquisition. Single B cells were identified with brightfield area close to 100, and aspect ratio close to 1 (Fig20 B). Focused cells were selected up to 60 brightfield gradient (Fig20 C). Double intensity of HLA and CD20 defined the B cell population (Fig20 D) and the intensity of donor-protein $CFSE^+$ cells were evaluated in the HLA-DR aspect ratio positive cells (Fig20 E).

After the population analysis, a cell-based analysis of positive images was performed. The surface of the cell was defined using the brightfield image. Then, CD20 (pink) and HLA-DR (red) molecules were identified on the surface of the B cells using both the intensity feature (for CD20 and HLA-DR together) and the brightfield image. Once the cell surface was delimitated by CD20 and HLA-DR intensity from their respective fluorescence channels, $CFSE^+$ donor-proteins, presenting a positive intensity in the FITC channel, were identified inside of the $CD20^+$/MHC class II^+ B cell (Fig20 F). To detect donor-proteins inside of the B cells, the image of the intensity of the $CFSE^+$ proteins was overlapped with the image of the intensity of $CD20^+$/MHC class II^+. A positive CFSE signal inside the B cells was observed, along with vesicle appearance, and these were found to be closer to the inner side of the membrane (Fig20 F). Cells incubated with autologous-proteins did not exhibit a CFSE signal **(-)** (Fig20 G) compared to cells incubated with allogeneic proteins **(+)** (Fig20 G).

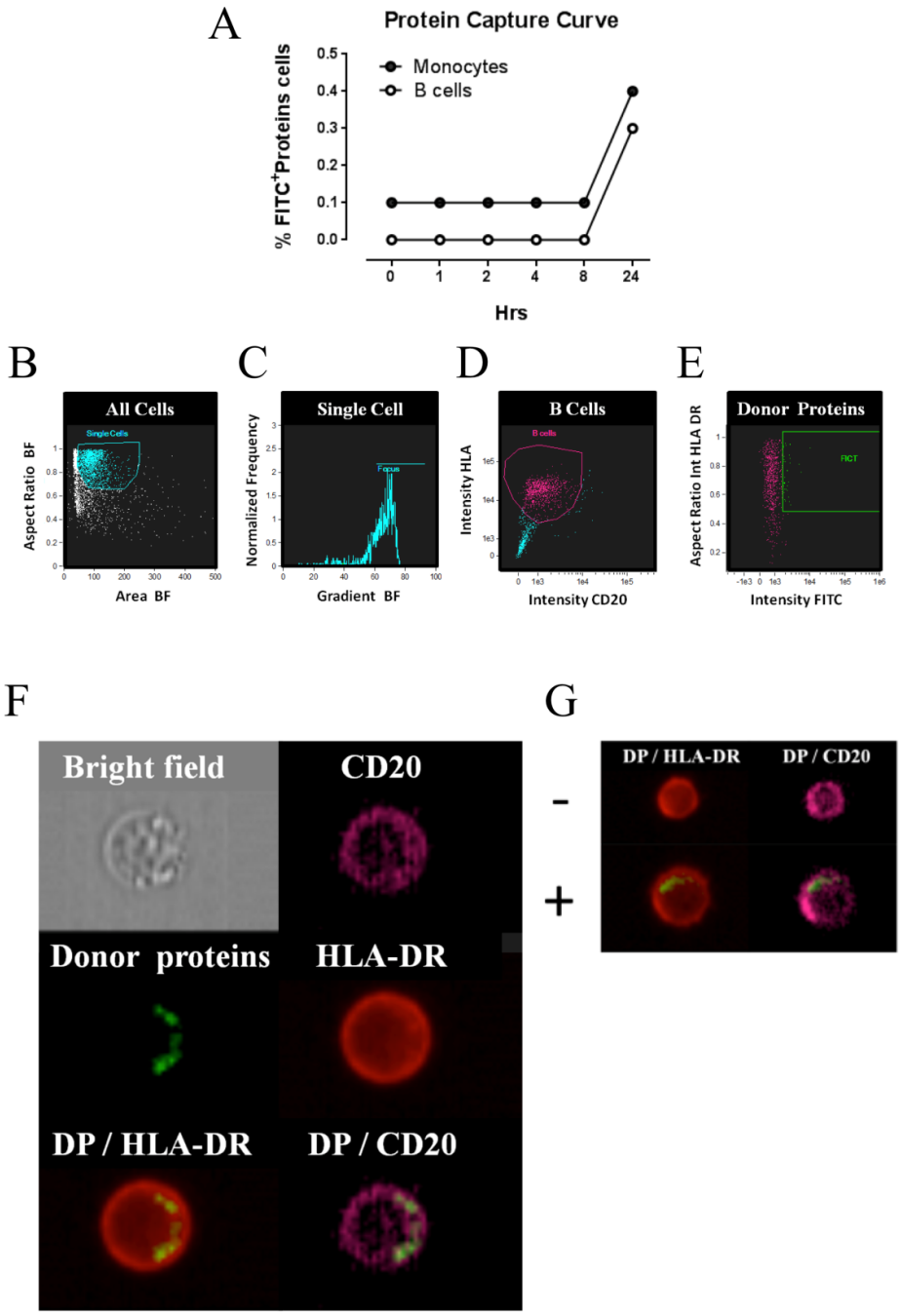


Figure 22: Donor-protein internalisation.

A protein-capture time course assay was performed after 1, 2, 4, 8, and 24 hours, to identify $CFSE^+$ cells within monocytes and B cells, when 0.5ug/ml of $CFSE^+$ proteins were cultured with $5.0x10^6$ PBMCS in a 12 well-plate with 4ml of complete media/well. (A). $CFSE^+$ B cells were enriched from the PBMCs and $CD20^+HLA\text{-}DR^+$ B cells were sorted before ImageStream analysis. Then, single cells were identified from the sorted population (B), focused cells were identified from the singles (C), B cells were identified from the focused cells (D) and $FITC^+$ B cells were identified from the B cell population (E). Images of bright field, CD20, HLA-DR, and $CFSE^+$ donor-proteins from sorted B cells were obtained using ImageStream (F). Negative (-) and Positive (+) controls of protein internalisation displayed by ImageStream images (G).

5.3.4 Donor-specific B cell presentation assays demonstrated a positive response in chronic rejector but not in tolerant and stable patients

The first aim of this experiment was to evaluate antigen capture by B cells from kidney transplant recipients by measuring $CFSE^+$ donor-protein internalisation by recipient B cells, using B cells as APCs and donor-proteins as allogeneic antigens. Allogeneic proteins (donor) and autologous proteins (recipient) were prepared and incubated with recipient PBMCs for 24 hours at 37°C. For each patient, the autologous protein capture (○—) and the allogeneic donor-proteins capture (—●) by $CD20^+$ B cells was measured using flow cytometry (Fig23 A). Firstly, only recipient B cells from chronic rejector exhibited a significant increase in protein recognition and internalisation of allogeneic donor-proteins (*p=0.0428). Secondly, donor-protein capture in B cells from chronic rejector was significantly higher than those from tolerant (***p=0.0002) and stable (****p<0.0001) patients.

The second aim of this assay was to analyse effector responses by measuring donor-specific T cell activation. Recipient $CD4^+$ cells were cultured with autologous B cell subsets loaded with donor-proteins. After 3 days of activation, $CD4^+$ T and $CD20^+$ B cells were harvested, CD25 expression was measured in $CD4^+$ T cells and CD86 was measured in $CD20^+$ B cell subsets, in order to study their expression after donor-antigen presentation. Surprisingly, CD25 expression by $CD4^+$ T cells was increased in tolerant patients (Fig23 B). All B cell subsets from all recipients expressed CD86 with equal intensity (Fig23 C).

After donor-specific activation, the $CD4^+$ T cell response was measured in an IFN-γ/IL-10 dual-ELISPOT assay. This method allowed the measurement of two cytokines at the same time in $CD4^+$:$CD20^+$ co-cultures activated with donor-proteins. The idea was to evaluate the Th1 response and the role of IL-10, in donor-specific activation. Background cytokine production from $CD4^+$:$CD20^+$ co-cultures without donor-proteins and from $CD4^+$ cultures with donor-proteins (without B cells) was subtracted from the cytokine production from donor-specific $CD4^+$:$CD20^+$ co-cultures. In healthy controls responses, background subtraction was exclusively from $CD4^+$ T cell cultures without B cells. IFN-γ and IL-10 spots were calculated per 100.000 $CD4^+$ T cells from the $CD4^+$:$CD20^+$ co-cultures.

Regarding the Th1 response, IFN-γ production by $CD4^+$ T cells (Fig23 D) induced by all B cell subsets was absent in tolerant samples. All stable samples secreted IFN-γ, but Naïve and Transitional subsets were the highest inducers. $CD4^+$ T cells from chronic rejector secreted IFN-γ, mainly from the sample activated by Memory B cells; Naïve B cells induced half of the amount present when $CD4^+$ T cells were co-cultured with Memory B cells, and Transitional B cells did not induce any IFN-γ production in this patient group.

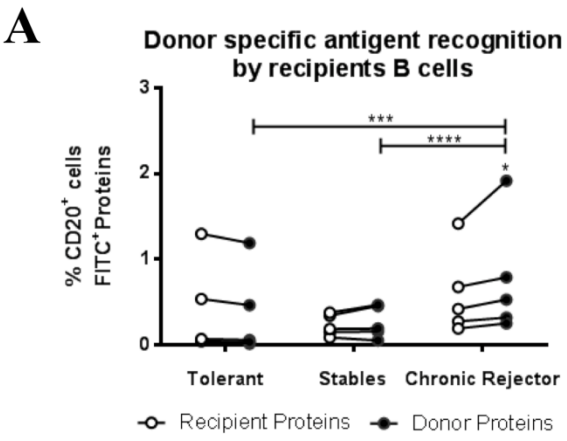
IL-10 secretion by $CD4^+$ T/$CD20^+$ cells (Fig23 E) was higher in healthy controls than patient samples, but no differences were observed between B cell subsets, although T cell cultures with Transitional B cells exhibited the highest frequency of IL-10 production. In tolerant and stable patients, cells did not secrete considerable amounts of IL-10. Interestingly, chronic rejector co-cultures secreted more IL-10 that other recipient samples, but all B cell subsets induced the same levels of this cytokine. It is unknown if the IL-10 secretion was coming from the B cell subsets or from the $CD4^+$ T cell population.

IFN-γ ■ and IL-10 □ spots from the IFN-γ/IL-10 dual-ELISPOT were plotted together to evaluate any relationship between the secretion of these two cytokines in healthy controls (Fig23 F) and kidney transplant patients (Fig23 G).

In healthy controls, both cytokines were identified in $CD4^+$ T cell co-cultures with all B cell subsets. In this group, IL-10 □ secretion was more than doubled compared to IFN-γ ■, and while Memory B cells induced higher levels of IFN-γ, Transitional B cells induced the highest levels of IL-10 (Fig23 F). $CD4^+$ T cells from tolerant patients failed to produce any IFN-γ despite clear evidence of $CD4^+$ T cell activation, whereas $CD4^+$ T cells from stable patients mainly secreted IFN-γ. $CD4^+$ T cells from chronic rejector secreted IFN-γ mainly from samples cultured with Memory B cells, and slightly less in T cells cultured with Naïve B cells. Interestingly, $CD4^+$ T cells cultured with Transitional B cells did not produce any IFN-γ (Fig23 G).

In conclusion B cells from tolerant recipients did not induce a Th1 response with any B cell subset, revealing a potential role for B cells in controlling the allogeneic-specific response to the graft. In chronic rejector, the groups with the highest protein capture, Memory B cells induced the higher Th1 response, followed by Naïve B cells,

which produced a median response. Interestingly, Transitional B cells did not induce IFN-γ production. The last result supports the idea that Transitional B cells do not activate $CD4^+$ T cell through the MHC-TCR donor-specific interaction.


A
Donor specific antigent recognition
by recipients B cells
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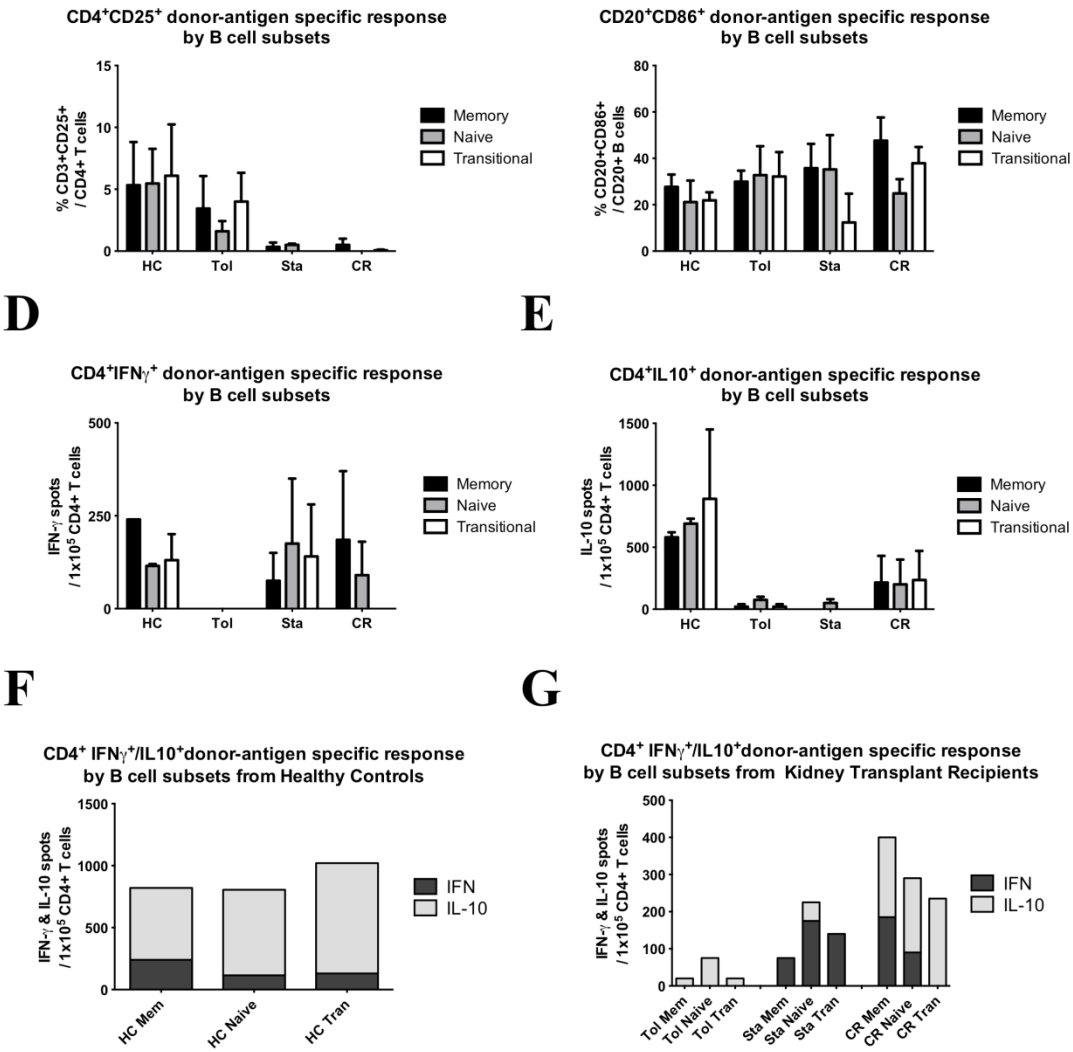

B
CD4+CD25+ donor-antigen specific response
by B cell subsets
% CD3+CD25+
/ CD4+ T cells
HC
Tol
Sta
CR
Memory
Naive
Transitional
C
CD20+CD86+ donor-antigen specific response
by B cell subsets
% CD20+CD86+
/ CD20+ B cells
D
CD4+IFNγ+ donor-antigen specific response
by B cell subsets
IFN-γ spots
/ 1x10^5 CD4+ T cells
E
CD4+IL10+ donor-antigen specific response
by B cell subsets
IL-10 spots
/ 1x10^5 CD4+ T cells
F
CD4+ IFNγ+/IL10+donor-antigen specific response
by B cell subsets from Healthy Controls
IFN-γ & IL-10 spots
/ 1x10^5 CD4+ T cells
HC Mem
HC Naive
HC Tran
IFN
IL-10
G
CD4+ IFNγ+/IL10+donor-antigen specific response
by B cell subsets from Kidney Transplant Recipients
Tol Mem
Tol Naive
Tol Tran
Sta Mem
Sta Naive
Sta Tran
CR Mem
CR Naive
CR Tran

Figure 23: Donor-specific antigen response

Internalisation of 0.1ug/ml of autologous ○— and allogeneic —● $CFSE^+$ proteins by $5.0x10^6$ $CD20^+$ B cells from kidney transplant recipients after 24 hours of culture, measured as $CD20^+CFSE^+$ B cells by flow cytometry (A). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used, * $p<0.05$ was considered significant. An IFN-γ/IL-10 dual-ELISPOT was performed to measured antigen-specific activation on $CD4^+$ T cells elicited by autologous B cells subsets from kidney transplant recipients. $1.0x10^5$ $CD4^+$ T cells were co-cultured with and without $0.5x10^5$ sorted autologous B cells subsets from healthy control (HC), tolerant (Tol), stable (Sta) and chronic rejector (CR) for 72 hours, with and without 0.1ug/ml of autologous or allogeneic $CFSE^+$ proteins. After 3 days, $CD4^+$ T cells and $CD20^+$ B cell subsets from the co-cultures were collected, and the expression of CD25 on $CD4^+$ T cells (B) and CD86 on the B cell subsets (C) were measured in the different groups of kidney transplant recipients using surface staining. IFN-γ (D) and IL-10 (E) production by $CD4^+$ T cell/$CD20^+$ B cell subsets co-cultures was measured using an IFN-γ/IL-10 dual-ELISPOT. Spots per 10^5 cells were calculated in each well after background deduction. Combined IFN-γ/IL-10 production was plotted in sample from healthy controls (F) and kidney transplant recipients (G). Two-way RM ANOVA test with a Tukey's multiple comparisons test was used, * $p<0.05$ was considered significant.

5.3.5 Donor-specific antibodies were more prevalent in serum samples from chronic rejector

HLA matching plays a fundamental role in the outcome of transplanted patients, particularly from deceased donors. Large national transplant databases clearly show that, in spite of improving immunosuppression, the higher the number of HLA antigen matches, the better the outcome following kidney transplantation, particularly in deceased donor transplants (Eremin *et al*. 2011).

In practice, renal transplant donors and recipients are matched in order to increase graft survival (Eremin *et al*. 2011). Three of the HLA loci are usually typed and matched in kidney transplantation: HLA-A, HLA-B, and HLA-DR. However nowadays, immunosuppressive drugs can mitigate the effect of HLA mismatching maintaining stable graft function even when more than one mismatch is present in the transplanted organ (Halloran 2004).

In this section, the relationship between HLA mismatches and the outcome in the different patients groups was investigated (Table 3). Interestingly, tolerant recipients had the lowest HLA mismatches; less than 50% for HLA-A, HLA-B and HLA-DR. More than the 70% of the stable patients presented at least one HLA mismatch in each locus. Patients in monotherapy varied; 50% had HLA-class I mismatch and less than 20% had HLA class-II mismatch. Chronic rejector were the group with the highest numbers of HLA mismatch, more than the 80% of the patients presented HLA differences with the donor, around 25% presented two mismatches and the rest presented only one mismatch (Table 3).

Donor-specific anti-HLA class-I and anti-HLA class-II antibodies were identified in serum samples from transplanted samples (Fig24). The number of total patients positive for DSA □, patients with HLA-I DSA ▭, patients with HLA-II DSA ■, and with both ⬚ were plotted. In the tolerant group, two patients presented HLA class-I DSA, and one patient HLA class-II DSA. The only stable patient positive for DSA had only one HLA class-I antibody. In the chronic rejector group, two patients presented HLA class-I DSA, four patients presented HLA class-II DSA, and a further five patients presented both.

In summary, chronic rejector were the patients with the highest number of DSAs. This suggests that the presence of these antibodies correlates with low graft survival and bad prognosis. However, two tolerant recipients also exhibited DSAs without any sign of chronic rejection, suggesting that DSAs may not be a reliable indication of graft lost. Conversely, this result may indicate that these tolerant patients are close of losing their graft and their tolerant state. Specificities in DSAs did not show any preference in participation of rejection or tolerance.

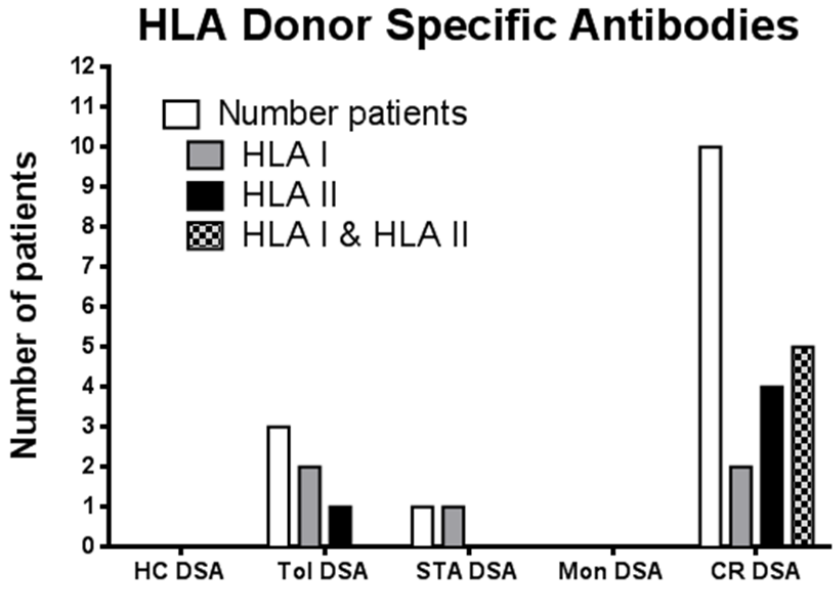


Figure 24: Donor-specific antibodies.

DSA were obtained from serum samples from healthy controls and kidney transplant patients. The total number of patients positive for total DSA ☐; the total number of patients positive for HLA-I DSA ▒; the total number of patients positive for HLA-II DSA ■; and total number of patients positive for HLA-I and HLA-II DSA ⊡ were plotted per patient groups.

5.4 Discussion.

5.4.1 Overview

The second chapter was designed to evaluate the BCR signalling pathway and the donor-specific responses elicited by B cells from the different patient groups. The first aim was to evaluate the function of the phosphorylation cascade of B cells from different patient groups by measuring phosphorylated proteins from the BCR signalling pathway after non-specific activation. Then, donor-specific assays and the measurement of anti-donor HLA antibodies were performed to understand the role of B cells as APCs in antigen-specific responses.

5.4.2 BCR activation

The BCR engagement is the main activation in the B cell population; when it binds the specific antigen it transduces the signal downstream through the BCR signalling cascade. In this pathway, several proteins are phosphorylated after BCR activation, one of which is ERK. The staining for ERK-p was very stable and was used as an indicator of general BCR activation in Memory, Naïve and Transitional B cells.

Irish *et al*. from Nolan's group studied the BCR-mediated signalling *via* phosphorylation of BTK, Syk, ERK1/2, and p38 in tumour B cells from follicular lymphoma samples (Irish *et al*. 2006; Irish *et al*. 2006). They found that p-ERK1/2 exhibited the highest activation signal after 8 minutes of activation with 10ug/ml of anti-IgM and anti-IgG $F(ab')_2$ fragments in $CD20^+$ cells within PBMCs. The same stimulus and kinetics of ERK and BTK reported by Nolan's group were used to design the activation protocol and the phospho-flow staining for this study.

Nolan's data reported that αIgM/αIgG was the best stimulus to activate the BCR in total B cells; to confirm this, IgM^+ and IgM^- B cells were studied in healthy controls and kidney transplant patient samples (Fig16). As expected $CD27^-$ B cells expressed high levels of IgM, and $CD27^+$ B cells exhibited both IgM^+ and IgM^- B cells. It was assumed that $CD27^+IgM^-$ B cells found in peripheral blood correspond to IgG expressing-B cells. A combination of anti-IgM/ anti-IgG was then used to activate both subsets at the same time. Concentration of anti-IgM/ anti-IgG was also a critical point in the experiments, as such, several concentrations of antibodies were tested and 20ug/ml

was found to be optimal for activation in all groups of patients.

Three proteins from the BCR pathway were evaluated in this study, BTK, BLNK and ERK, but only ERK was stable enough to be measured in patient samples. In addition, several time points were evaluated, and it was found that 10 minutes was sufficient to identify ERK-p with a fair separation between the activated and the non-activated sample. Nolan's group also reported a shorter time of activation and a lower intensity in BTK-p compared to ERK-p, a result that was also found in this study during the standardisation; however this result is explained by the fact that BTK-p appears much earlier than ERK-p in the BCR signalling cascade.

ERK-p levels were studied after BCR, CD40 and BCR/CD40 activation, but only BCR activation induced a positive response. Even when both activations (BCR and CD40) were performed together, CD40-CD40L interaction decreased BCR activation instead of promoting a higher response. Contrary to the data from this thesis, Ying *et al*. examined whether CD40 ligation activated the BCR signalling pathway (Ying *et al*. 2011). They stimulated BALB/c splenic B cells with anti-IgM $F(ab')_2$, anti-CD40, or both, and blotted with phospho-antibodies against Lyn, Syk, and BLNK. They found that CD40 stimulation induced phosphorylation of Syk and BLNK, whereas BCR and CD40 co-stimulation resulted in a synergistic activation of Syk, but not Lyn. Their results suggest that CD40 activation shares components of the BCR signalling pathway, and because of that, CD40 may quantitatively enhance BCR-mediated signalling. They also found that BTK-p and ERK-p were enhanced by CD40 co-stimulation in BCR-mediated signalling (Mizuno *et al*. 2005; Ying *et al*. 2011); interestingly B cells were activated first *via* CD40, and then by using anti-IgM (Mizuno *et al*. 2005). The main differences between Ying's study and this thesis were the origin of the cells and the technique used to detect phosphorylation; this study used PBCMs and a phospho-flow, and they used BALB/c splenic B cells and western blot. They also added an anti-mouse CD40 activating antibody, while CD40L-transfected L cells were used in this study. Possible reasons for these discrepancies include: western blot is more sensitive than phospho-flow to detect phosphorylation signals; CD40L-transfected L cells were not added at the right concentration; B cells from the spleen responded better to CD40 activation than B cells from peripheral blood, a different proportion of Naïve and Memory in the sample; or simply because as PBMCs already presented the CD40 stimulus needed to activate B cells, the addition of CD40L-transfected L cells was

irrelevant in the activation system, and they only interfere with the BCR engagement due their large size.

Finally, Duddy *et al*. demonstrated that B cells exhibited different responses, depending on the activation received (CD40, BCR, BCR+CD40) (Duddy *et al*. 2004). Whereas CD40 activation induced an anti-inflammatory response, BCR activation induced a pro-inflammatory response that was enhanced with the CD40-CD40L interaction. These results suggests that in human B cells, BCR and CD40 activation trigger different signalling pathways, and that BCR activation can be boosted by CD40 co-stimulation.

In conclusion, ERK-p evaluation allowed the measurement of BCR activation in Memory, Naïve and Transitional B cells. This activation was short, non-specific, and controlled by several factors. The main benefit of this technique was the possibility of measuring BCR activation in different B cell subsets within PBMCs.

5.4.3 ERK-p was detected in the B cell subsets by phospho-flow

Phospho-flow was used to identify ERK-p in B cell subsets within PBMCs. The challenge was to distinguish Memory, Naïve and Transitional B cells, get a fair viability and avoid complex sample preparation. PBMCs were used because, after several tests, it was ascertained that B cells maintained a better viability when they were not isolated, as isolated or sorted B cells exhibited a low survival rate (data not shown).

Regarding the use of phospho-flow panels in transplantation, Silva *et al*. found significantly higher levels of STAT3-p in B cells from kidney transplant tolerant recipients after CD40 activation (Silva *et al*. 2012). The same technique was used before by Claudia Mauri's group to study the IL-10 pathway after CD40-CD40L interaction (Blair *et al*. 2010). The results demonstrated a relationship between tolerance and IL-10 signalling. Both groups identified that the main STAT-3-p expressing cells were within the Transitional B cells.

Although identification of the link between tolerance and the Transitional population through STAT3-p was of interest, the phospho-panel was used in this chapter to study the BCR activation and donor-specific responses rather than CD40 activation. Activation *via* CD40 was measured in the previous chapter with a different

method.

5.4.4 B cell subsets from tolerant recipients displayed a defective BCR signalling pathway compared to healthy volunteers

BCR activation was measured in total B cells and B cell subsets from healthy controls and kidney transplant patients. These experiments generated two important results: total B cells and B cell subsets from tolerant recipients failed to phosphorylate ERK after BCR activation, and Transitional B cells exhibited lower ERK-p than Memory and Naïve B cells.

DeFranco's group established that BCR activation varies depending on the maturation state of the cell and on the additional signals that the cell receives (DeFranco 2000). They proposed that antigen-BCR interaction in immature mouse B cells typically causes them to enter into an anergic state or undergo apoptosis. In contrast, mature B cells coming into contact with antigens enter the G_1 phase of the cell cycle and up-regulate many proteins involved in adhesion and antigen presentation. Strong BCR stimulation can also induce differentiation of B cells into antibody-secreting Plasma cells by providing cell-cell contact signals *via* CD40, and by releasing cytokines such as IL-4 and IL-5 (DeFranco 2000; Richards *et al*. 2001). These different responses can be associated with the ERK-p observed in B cell subsets from this thesis, as Transitional B cells had lower responses than Memory and Naïve B cells.

Another group reported in a mouse model that BCR ligation on immature B cells induced apoptosis, whereas on mature B cells induced cell activation and growth (Koncz *et al*. 2002). They compared the kinetics of ERK activation in transitional immature and mature B cells and found that in immature B cells, ERK was transiently phosphorylated, while in mature cells, ERK phosphorylation was sustained up to 2 hours. The lack of sustained ERK activation in immature B cells suppressed the transcription factors necessary for proliferation signals (Koncz *et al*. 2002).

Another study also reported the role of ERK in B cell survival by comparing BCR with BAFF activation. They found that although BCR cross-linking induced rapid activation of ERK within 30 minutes, ERK activation subsequently declined below basal levels within 16 hours and remained low. On the other hand, BAFF induced delayed but sustained stimulation of ERK. Their results suggested that BAFF inhibited

death by sustained ERK activation (Craxton *et al*. 2005), as a consequence, they consider ERK activation as an indicator of better B cell viability.

A case report showed that T1 and T2 B cells in mice were more sensitive to BCR stimulation than mature follicular B cells for BCR-induced ERK phosphorylation. This phenomenon was explained by the absence of Lyn (a kinase that phosphorylates ITAMs on the BCR Igα/Igβ chains following antigen binding) in the immature population. Then, while the B cell signalling was greatly enhanced in mature B cells, the B cell signalling was minimally affected in the immature cells (Gross *et al*. 2009). These results in mouse models suggest that mature B cells responded better than immature B cells to full BCR activation.

In studies with human cells, Ha *et al*. compared BCR activation in B cell subsets from cord blood, bone marrow, and peripheral blood. First, they investigated the activation of ERK and p38 in B cells following stimulation with anti-IgM or anti-CD40 antibodies. Then, they identified different B cell subsets with IgD, CD27, CD24 and CD38 markers, and finally they measured ERK and p38 phosphorylation in these well-defined B cell subsets. They found that B cells in cord blood contained an increased proportion of CD24-expressing B cells and a low proportion of CD27-expressing B cells. In addition, a minimal CD40-mediated activation of ERK and p38 was observed in these cells (Ha *et al*. 2008), implying that Transitional B cells were not responding to BCR activation.

In the study presented in this thesis, B cells from tolerant recipients failed to be activated *via* BCR compared to B cells from healthy controls. This result was important because for the first time a difference was observed between B cells from tolerant patients and healthy controls. This result suggests that B cells from tolerant recipients revealed a certain degree of anergy or unresponsiveness; interestingly, this effect was also observed in Memory and Naïve B cells from tolerant recipients. This could imply that total B cells from tolerant recipients enter into a non-responsive state after BCR activation.

Looking at the different B cell subsets, it was observed that in healthy controls, a clear pattern could be established in which Memory B cells were the main responders to BCR activation, Naïve B cells were intermediate and Transitional B cells were the lowest responders. This pattern was lost in all patients groups except in chronic rejector.

All these data together suggest that B cells could contribute to tolerance *via* failure in the BCR activation.

5.4.5 Donor-specific responses overview

Several reports support the idea that whereas direct recognition plays a dominant role in acute rejection, indirect recognition is the main pathway involved in chronic allograft failure (Mason *et al*. 1996; Hornick *et al*. 2000; Baker *et al*. 2001; Baker *et al*. 2001). This is because T cells require co-stimulatory signals from specialised APCs to get activation. In the case of direct recognition, within hours/weeks of transplantation, T cells are activated by the recognition of intact foreign MHCs on the surface of donor APCs from a transplanted organ, particularly DCs. After few weeks from transplantation, DCs of donor origin are depleted, therefore the occurrence of direct recognition is unlikely (Game *et al*. 2002). In the case of indirect recognition, when donor MHC are internalised, processed, and presented as peptides by host APCs, a permanent activity is observed after transplantation due to a continual trafficking of recipient APCs through the graft (Game *et al*. 2002).

Pathways of allorecognition also have implications in transplantation tolerance. In order to obtain clinical tolerance, an induction of specific tolerance in T cells with indirect allospecificity is required to control the recipient immune response (Game *et al*. 2002). B cells are professional APCs; they express MHC class II and can therefore elicit indirect responses through donor-peptide presentation in the context of MHC class II. One of the aims of this thesis was to evaluate the induction of indirect alloresponses by T cells, induced by donor-specific B cell and their subsets. The final goal was to find a relationship between these cellular responses and the clinical responses in kidney transplanted patients.

5.4.6 Donor-protein preparation and internalisation

To develop donor-specific assays, a new method was set up to obtain soluble proteins from donor cells. This protocol has evolved from protocols previously reported (Baker *et al*. 2001; Bestard *et al*. 2008; Nickel *et al*. 2009). The aim of this part of the project was to measure donor-proteins inside of the B cells, thus the first goal was to obtain stained soluble donor-proteins identifiable by flow cytometry. MHC molecules conjugated with fluorescent proteins were available (as the ones used to identify donor-

specific antibodies by Luminex kits), however, the cost was very high and it was impossible to obtain them separately. Furthermore, even if common HLA-A/B/C and HLA/DR molecules were used, DQ, DP, mH and other relevant proteins would still be missing in the assay. For all these reasons, a new method was set up using a CFSE staining in the donor $CD2^-$ cells before the membrane and cytosolic protein extraction. This technique allowed obtaining a variety of $CFSE^+$ donor-proteins, identifiable by flow in the FITC channel, from several donor samples.

5.4.7 Control samples used in donor-specific activation assays

Two samples were used as controls, the first sample derived from a woman that had three pregnancies with the same partner. It is known that mammalian reproduction poses an immunological paradox because foetal alloantigens encoded by genes inherited from the father, should provoke responses by maternal T cells leading to foetal loss (Mellor *et al*. 2000). One of the hypotheses to explain foetal tolerance proposed that the foetus is not recognised as foreign by the mother; however, women who have several children with the same partner usually make alloantibodies recognising the partner MHC mismatched molecules. Indeed, this has been recognised as the best source of antibodies for human MHC typing (Murphy *et al*. 2012). The existence of antibodies indicates the existence of paternal specific B cells.

In 1997, Susuki *et al*. reported pooled results of spousal donor transplants performed at 97 centres in the USA. The data was gathered from 250 centers in the USA in the UNOS Registry between October 1987 and December 1996, and analysed by Kaplan-Meierb survival curves. The authors of the study investigated the effect of pregnancies on transplant outcome (Picture 7) and found that wives with a single previous pregnancy had a high graft survival rate comparable to those with more than one pregnancy. Based on these results a spouse/wife (with three pregnancies) was used to increase the possibility of identifying donor-specific B cells.

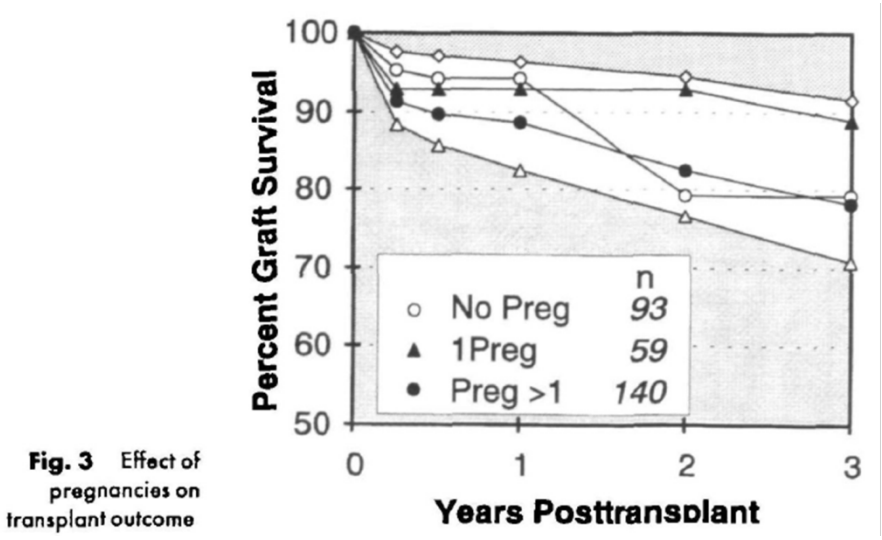


Fig. 3 Effect of pregnancies on transplant outcome

Picture 7: Effect of pregnancy in graft survival

Figure extracted from "Unrelated living donor kidney transplants" (Suzuki *et al*. 1997).

When the B cells from the former control sample and the second control sample (that corresponded to a kidney transplant recipient with known HLA alloantigens recognised) were incubated with CFSE labelled "donor" proteins, a high background in the staining was observed in positive samples compared to the unstained samples. Therefore, a sample without $CFSE^+$ proteins was not the proper negative control to obtain percentages of $CFSE^+$ B cells. The problem was solved using as a sample of self-$CFSE^+$ proteins as a negative control (knowing no history of autoimmune disease in both cases).

Although this data showed CFSE-$proteins^+$ B cells, and a difference between the negative and positive control, protein internalisation was not proven because donor-antigens are recognised by the BCR, therefore the signal obtained could have corresponded to a surface location instead of an internal one. ImageStream was used to confirm the location of $CFSE^+$ donor-proteins inside the B cells.

Finally, it was concluded that protein recognition and internalisation was assessed with this method and the next step was to measure both parameters in patient samples.

5.4.8 Donor-specific activation exhibited by kidney transplant patients

Recipient B cells were cultured with protein preparations from recipient and donor samples to identify antigen recognition and internalisation in different patient groups. Results demonstrated that B cells from chronic rejector internalised more donor-proteins compared to tolerant and stable patients.

The donor-specific indirect response was then observed using recipients $CD4^+$ T cells as responders, donor-proteins as antigens, and B cell subsets as APCs. The main idea of this experiment was to evaluate the contribution of each B cell subset to donor-antigen presentation.

Chung *et al*. investigated the ability of murine Transitional B cells to process and present antigens to $CD4^+$ T cells, and to elicit protective signals in the absence of CD86 up-regulation. They reported that Transitional B cells processed and presented antigens as peptide:MHC class II complexes, but their ability to activate T cells and provide help to $CD4^+$ T helper cells was compromised (compared to Mature B cells) unless exogenous T cell co-stimulation was provided. Their data suggest that during an immune response, Transitional B cells may be able to sustain the responses of pre-activated $CD4^+$ T cells, but are unable to initiate activation of Naïve T cells (Chung *et al*. 2003). This report demonstrated the functional role of Transitional B cells as APCs due to their ability to process and present donor-antigens.

In this thesis, results obtained from the control samples exhibited antigen internalisation in all B cell subsets, including Transitional B cells (data not shown). However, B cells from tolerant recipients did not show any $CFSE^+$ B cells within any of the B cell subsets analysed. The fact that tolerant recipients had more HLA-matches with their respective donors compared to stable patients and chronic rejector, could possibly have affected the percentage of B cells capable of capturing donor-proteins. This could be due to a lower diversity of proteins present in preparations from donors of tolerant recipients; data from dual-ELISPOT support this result as IFN-γ was not secreted by responders $CD4^+$ T cells co-cultured with B cell subsets from tolerant recipients.

In contrast, B cells from chronic rejector demonstrated a positive donor-protein capture and a positive antigen-presentation to $CD4^+$ T cells, as demonstrated in the dual-ELISPOT results. Interestingly, in this patient group, Memory B cells were the best APCs in the induction of the Th1 response, whereas Naïve B cells demonstrated a lower

potential in the induction of IFN-γ. Transitional B cells did not induce any IFN-γ production by $CD4^+$ T cells.

B cells from stable patients did not show a clear capture pattern, and Naïve and Transitional B cells induced more Th1 response than Memory B cells.

In conclusion, only data obtained from the analysis of chronic rejector agreed with Chung's suggestion that Transitional B cells are unable to activate T cells. Regarding the results in tolerant recipients, Haynes *et al*. investigated the role of the donor-specific indirect responses from T cells in renal transplant tolerance using *trans-vivo* delayed-type hypersensitivity (tvDTH). They found that the indirect pathway was active in tolerant, steroid-monotherapy, standard immunosuppression and chronic rejection patients, but not in identical twin recipients. Although they found responses in all patients groups, distinct intergroup differences were evident and they corresponded to the clinical status (Haynes *et al*. 2012) They found that tolerant recipients exhibited the lowest response, followed by patients in monotheraphy, then stables, and finally, chronic rejector. These findings concur with the results obtained from tolerant recipients in this thesis, as they did not induce a pro-inflammatory response. Haynes *et al*. also tested blocking antibodies in their tvDTH model to evaluate the participation of IFN-γ, TGF-β, IL-17 and IL-10. Whereas anti-IFN-γ and anti-IL-17 antibodies significantly blocked the indirect pathway of T effector cell responses to donor-antigens in the chronic rejector patients, antibodies against TGF-β blocked the suppressive response observed in tolerant recipients. In contrast, IL-10 did not appear to contribute to the anti-donor cell response, as neutralising IL-10 did not affect the footpad swelling to donor-antigen. The results of this thesis did not demonstrate any clear involvement of IL-10 in antigen-specific responses. IL-10 was mainly produced during co-cultures of B and T cells obtained from chronic rejector, but no differences between B cell subsets was observed within this group. Finally, they demonstrated the activity of B cells in the tvDTH assay. They depleted B cells from one stable and one tolerant patient and they measured the inhibitory response to allo-peptides. Neither patient's inhibitory response was affected by depletion or reintroduction of B cells. Even when no clear B cell participation in tolerant patients was identified, a link between tolerance and B cells was suggested, as two of the three tolerant patients with the lowest regulation to donor-antigen also had the lowest number of B cells.

In summary, like T cells, B cells cannot be considered as cells with one specific function. Similar to T cells that can have effector, regulatory and cytotoxic functions, B cell subsets also seem to have different responses and roles in the immune system.

Transitional B cells have been the main B cell population related to tolerance in humans; for example, patients with severe immunodeficiency exhibited an increment of circulating immature/transitional B cells with a noticeable reduction of circulating memory B cells (Martinez-Maza *et al*. 1987; De Milito *et al*. 2001; Cuss *et al*. 2006). Blair *et al* demonstrated the importance of Transitional B cells in human autoimmune disease, showing that patients with systemic lupus erythematous were refractory to CD40 stimulation, and therefore, the suppressive capacity of the B cells was impaired when IL-10 pathway was blocked (Blair *et al*. 2010). Finally, a new intriguing role of Transitional B cells in kidney transplantation was revealed since tolerant recipients exhibited an expansion of B cells, especially Transitional B cells in peripheral blood (Newell *et al*. 2010; Sagoo *et al*. 2010; Silva *et al*. 2012). All these clinical data support the idea that Transitional B cells exhibit a specific regulatory role able to modulate different immunological processes *via* different mechanisms.

5.4.9 Donor-specific antibodies were more prevalent in serum samples from chronic rejector

Although several studies reported the prevalence of donor-specific antibodies (DSAs) in chronic rejection (Kimball *et al*. 2011; Loupy *et al*. 2012; Mohan *et al*. 2012; Page *et al*. 2012) the role of DSAs in mediating chronic allograft injury is not fully understood. Hidalgo reported that in addition to the detrimental effect of pre-existing DSAs in the outcome of the transplant, *de novo* antibodies, emerging at any time after transplantation, could also negatively affect graft survival (Hidalgo *et al*. 2009).

Hidalgo also reported that DSAs have different pathogenicity. Recent observation supported the idea that *de novo* DSAs are mainly specific to donor MHC class II; interestingly, DSAs against MHC class II are associated with a worse prognosis than DSAs against MHC class I (Hidalgo *et al*. 2009). In this study a mixture of MHC class I and II DSAs were found in all kidney transplant recipients, except in monotherapy. These results suggest that MHC class I and II are not only participating in the immunological response in transplantation during rejection.

Incidence of DSAs was clearly higher in patients with chronic rejection. This was not only found in the numbers of patients positive for DSAs, it was also seen in the number of antigenic specificities shown by recipients from this group. This observation supports the results from the antigen capture assays where the percentages of $CFSE^+$ B cells from chronic rejector were higher compared to tolerant and stable recipients. Interestingly, two tolerant recipients also presented DSAs without any clinical signs of possible rejection.

In summary the role of antibodies in transplantation tolerance is not fully understood with this small numbers of samples. It was concluded that because DSAs are prevalent in chronic rejection, they contribute to this clinical state, however it was observed that the presence of DSAs does not always induce rejection, since two tolerant recipients with stable graft function were positive for the presence of DSAs.

6 The effect of B cell subsets as $CD4^+$ T cell-activating cells

6.1 Introduction.

The cross-talk between B and T cells, that involves specific antigen recognition, and protein-protein interaction, can lead to cell activation and cytokine production (Clark *et al*. 1994). The TCR and the MHC are the main players in cognate recognition. The TCR recognises specific self-restricted peptides in the context of the MHC molecules. Although TCR:peptide-MHC complex is an important interaction, participation of co-stimulatory molecules is essential to induce cell survival and/or to lead to different activation pathways. Co-stimulation molecules induce different responses: CD80/CD86-CD28 induces T cell activation, CD40-CD40L induces B cell survival and maturation, and CD86-CTLA-4 induces anergy (Dong *et al*. 1999). Cytokines also play an important role in the cross-talk between cells; they can induce cell survival, proliferation, activation, differentiation or suppression depending of the type of cytokine released.

In the following set of experiments, the incidence of B cells in the induction of proliferation, activation and cytokine secretion by $CD4^+$ T cell was evaluated. B cell subsets were isolated from peripheral blood samples (healthy volunteers), and co-cultured with autologous $CD4^+$ T cells to evaluate for each subset, the potential role as $CD4^+$ T-activating cells.

Memory, Naïve and Transitional B cells not only exhibit a specific phenotype, they also express a different array of co-stimulatory molecules, distinct cytokine secretion profiles, and different survival properties. These differences between the subsets are the main causes of the variety of, and sometimes opposite, effects in $CD4^+$ T cell proliferation, activation and cytokine secretion.

In summary, this chapter was designed mainly to answer the last hypothesis in this thesis:

- Transitional B cells exhibit an anti-inflammatory response compared to Naïve and Memory B cells.

6.2 $CD4^+$ T cell activation by B cell subsets.

6.2.1 Memory, but not Transitional B cells induced $CD4^+$ T cell proliferation after five days of co-culture

In order to understand the potential role of B cells as $CD4^+$ T-activating cell, $CD4^+$ T cell proliferation induced by autologous B cell subsets was studied. T and B cells were obtained from healthy volunteer's blood samples. B cell subsets were then isolated to study their distinctive T cell-activating effects. $CD4^+$ T cells were activated with an anti-CD3 antibody in order to up-regulate CD40L expression on the T cell surface, and to consequently activate the B cells through CD40-CD40L interaction. Expression of CD40L was measured on the T cell surface after 12 and 24 hours of anti-CD3 activation, and maximal CD40L expression was obtained between 6 and 12 hours of activation (Fig25 A). To increase chances of CD40-CD40L interaction, B cells were then added after 6-8 hours of $CD4^+$ T cell activation (when CD40L expression was at the highest level). To allow this interaction, $CD4^+$ T cells were activated while B cell subsets were isolated by flow sort, allowing both cells to be ready at the same time before the five-day co-culture.

After T and B cells were co-cultured, a time course was performed to evaluate T cell proliferation rates at day 1, 3 and 5 induced by Memory, Naïve and Transitional B cells (Fig25 B). Proliferation started after 3 days of co-culture and by day 5, Memory B cells induced the highest $CD4^+$ proliferation rate compared to the other B cell subsets.

Once it was established that day 5 was the optimal time to measure proliferation, a new large-scale experiment was undertaken to measure, in the T-B cell co-cultures, cell proliferation, expression of activation markers and cytokine production (Fig25 C). This experiment had two new conditions (compared to the time course experiment); $CD4^+$ T cells alone to measure the basal proliferation rate, and $CD4^+$ T cells with total B cells (33.3%/each subset) to control T cell proliferation in the presence of total B cells. Results showed that $CD4^+$ T cells cultured with Memory B cells significantly increased proliferation rates compared to $CD4^+$ T cells cultured alone (*p=0.0169), or with Transitional B cells (*p=0.0146). However, no difference was observed in the proliferation of $CD4^+$ T cells co-cultured with Naïve B cells. Therefore, it can be suggested that Transitional B cells failed to stimulate proliferation of $CD4^+$ T cells.

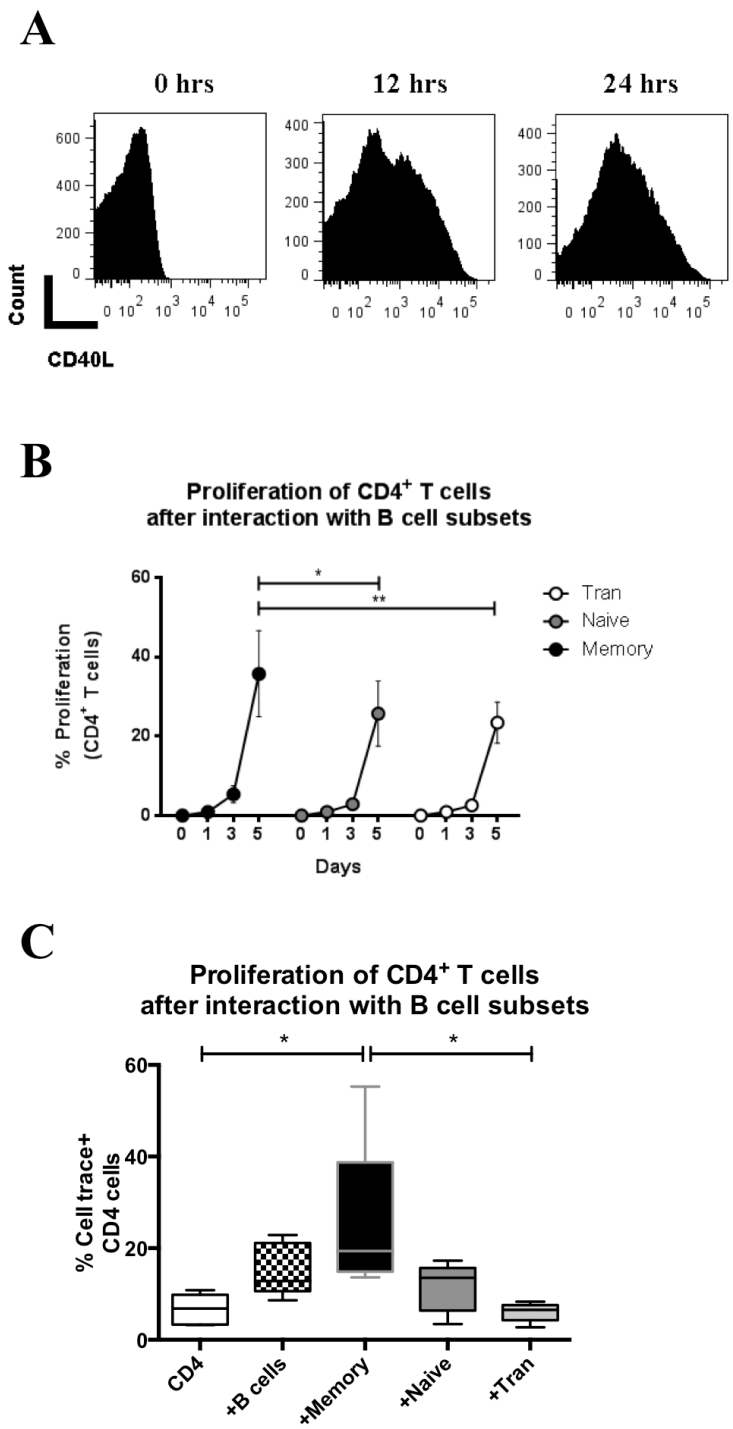


Figure 25: CD4$^+$ T cell proliferation induced by B cell subsets.

Expression of CD40L was measured in the cell surface of 1.0x10^6 CD4$^+$ T cells after 0, 12 and 24 hours of activation with anti-CD3 (1ug/ml, plate-bound) (A). A time course was performed to measure T cell proliferation in co-cultures of 0.5x10^6 anti-CD3-activated CD4$^+$ T cells with 0.5x10^6 Memory, Naïve or Transitional B cells after 0, 1, 3 and 5 days of co-culture (B). The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Tukey's multiple comparisons test. Percentages of T cell proliferation were measured in co-cultures of 1.0x10^6 anti-CD3-activated CD4$^+$ T cells with or without 0.5x10^6 B cells, Memory, Naïve or Transitional B cells after 5 days of co-culture (C). The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.2.2 Transitional B cells did not induce expression of CD25 and CD69 molecules on $CD4^+$ T cells compared to Memory B cells after five days of culture

Expression of activation molecules CD25 and CD69 were measured by surface staining on $CD4^+$ T cells co-cultured with total B cells and B cell subsets, after 5 days of culture. These proteins were measured as an indication of T cell activation induced by B cell subsets.

Results showed that CD25 was significantly up-regulated in $CD4^+$ T cells cultured with Memory B cells compared to $CD4^+$ T cells cultured alone (*p=0.0260) or with Transitional B cells (*p=0.0350) (Fig26 A).

In terms of CD69, it was found that this marker was also significantly increased in $CD4^+$ T cells cultured with Memory B cells compared to $CD4^+$ T cells cultured alone (*p=0.0499) or with Transitional B cells (*p=0.0152) (Fig26 B). There was also a significant increase of CD69 expression in $CD4^+$ T cells cultured with Memory B cells compared to $CD4^+$ T cells cultured with Naïve B cells (*p=0.0192) (Fig26 B).

Altogether Memory, but not Transitional, B cells were able to induce up-regulation of CD25 and CD69 in autologous $CD4^+$ T cells after five days of co-culture.

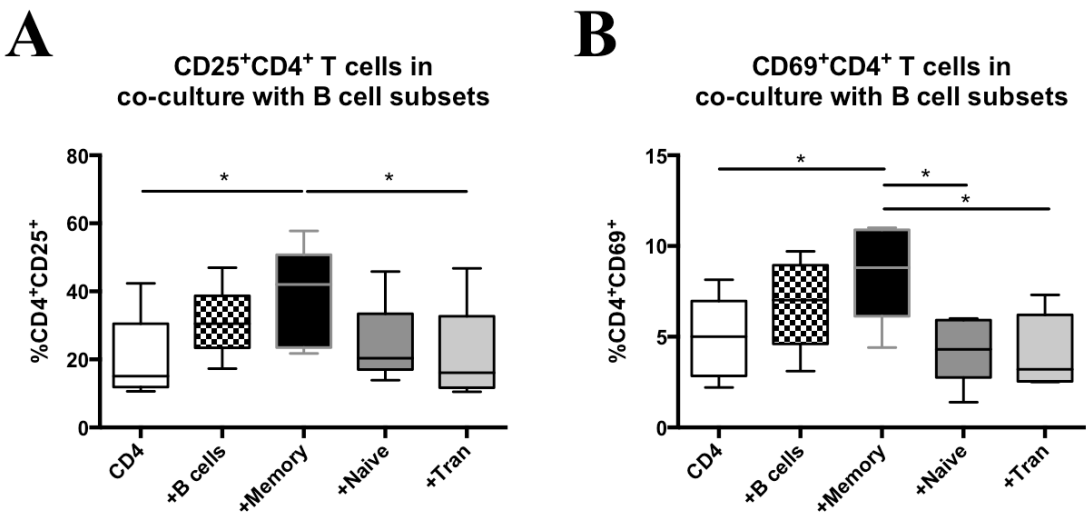


Figure 26: Activation molecules in $CD4^+$ T cells co-cultured with B cells.

CD25 (A) and CD69 (B) expression was measured in $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells, co-cultured with or without $0.5x10^6$ B cells, Memory, Naïve or Transitional B cells after 5 days using surface staining. The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.2.3 Transitional B cells did not induce production of pro-inflammatory cytokines by $CD4^+$ T cells compared to Memory B cells after five days of culture

Pro-inflammatory cytokine production was measured in activated $CD4^+$ T cells co-cultured with or without B cells and B cell subsets after 5 days of culture; IFN-γ and TNF-α were measured using intracellular staining and ELISA.

Similarly to proliferation and expression of activation markers, Memory B cells were, once again, the main activating population in terms of cytokine production. A higher percentage of $CD4^+IFN\text{-}\gamma^+$ T cells was found in co-cultures of T cells with Memory B cells, compared to $CD4^+$ T cells alone (*p=0.0122) or with Transitional B cells (**p=0.089) (Fig27 A). Results obtained with ELISA revealed that $CD4^+$ T cells co-cultured with Memory B cells also induced higher levels of IFN-γ, compared to $CD4^+$ T cells cultured alone (**p=0.0069) or with Transitional B cells (*p=0.0183). Co-cultures of $CD4^+$ T cells with total B cells also exhibited a significant increase in IFN-γ production compared to $CD4^+$ T cells cultured alone (**p=0.0078) or with Transitional B cells (*p=0.0201) (Fig27 B). This implies that IFN-γ induced by total B cells is mainly coming from the activation with the Memory population.

TNF-α, the second pro-inflammatory cytokine studied, was also measured by intracellular staining and ELISA. In the first case, high percentages of $CD4^+TNF\text{-}\alpha^+$ T cells were found in co-cultures with Memory B cells, compared to $CD4^+$ T cells alone (*p=0.0282) or with Transitional B cells (*p=0.0282) (Fig27 C). In the second case, $CD4^+$ T cell co-cultures with Memory B cells secreted significantly higher levels of TNF-α, compared to $CD4^+$ T cells cultured alone (**p=0.0028) or with Transitional B cells (*p=0.0144). Co-cultures of T cells with total B cells also significantly increased TNF-α production, compared to $CD4^+$ T cells cultured alone (*p=0.0255) (Fig27 D).

In summary, Memory B cells were the main population that induced pro-inflammatory cytokine production by $CD4^+$ T cells. Naïve B cells also participated in cytokine production, but at a lower scale, and Transitional B cells did not induce pro-inflammatory secretion at all.

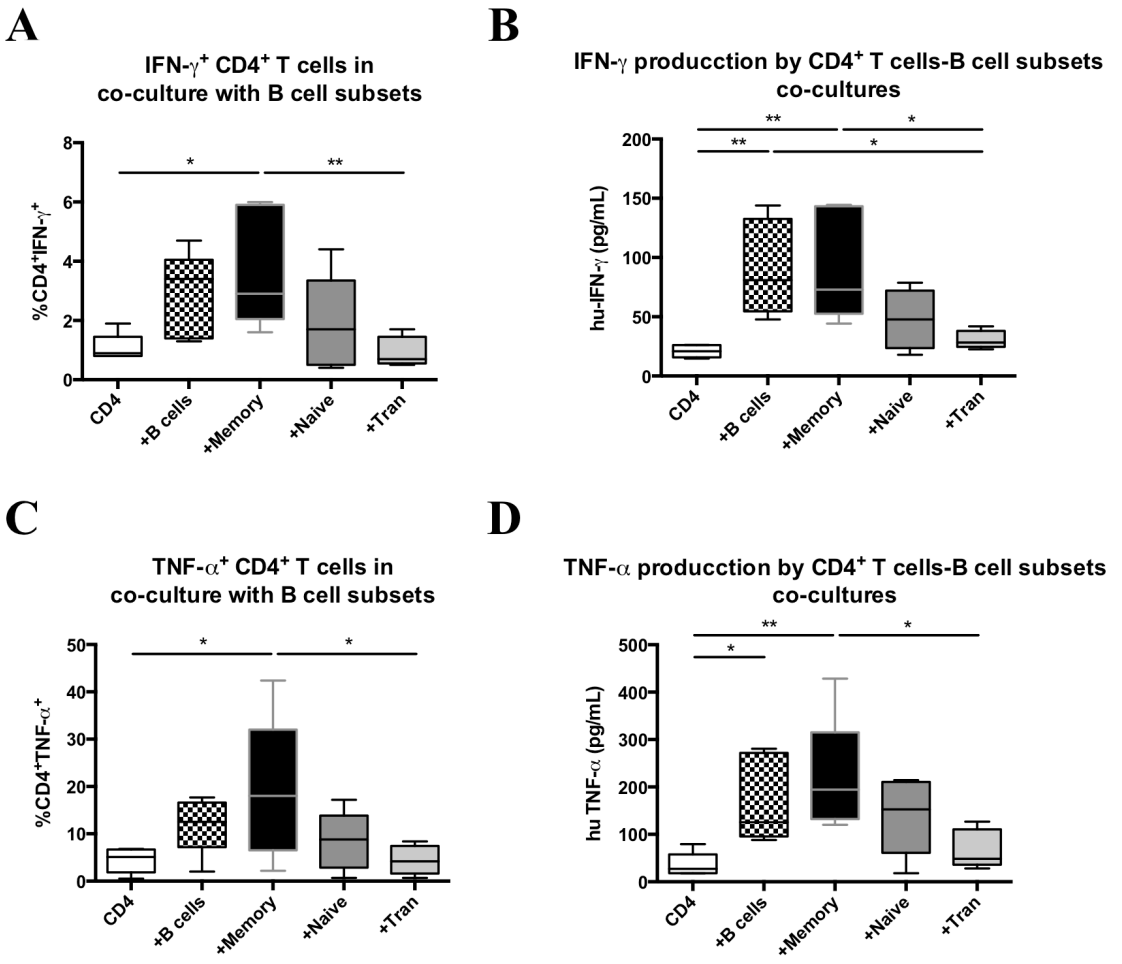


Figure 27: Identification of pro-inflammatory cytokines produced by $CD4^+$ T cells co-cultured with B cells.

Percentage of $CD4^+IFN\text{-}\gamma^+$ cells (A) and levels of IFN-γ (B) from co-cultures of $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells with or without $0.5x10^6$ B cells, Memory, Naïve or Transitional B cells after 5 days of co-culture. Percentage of $CD4^+TNF\text{-}\alpha^+$ cells (A) and levels of TNF-α (B) from co-cultures of $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells with or without $0.5x10^6$ B cells, Memory, Naïve or Transitional B cells after 5 days of co-culture. The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.3 Role of Memory, Naïve and Transitional B cells as $CD4^+$ T cell activating cells.

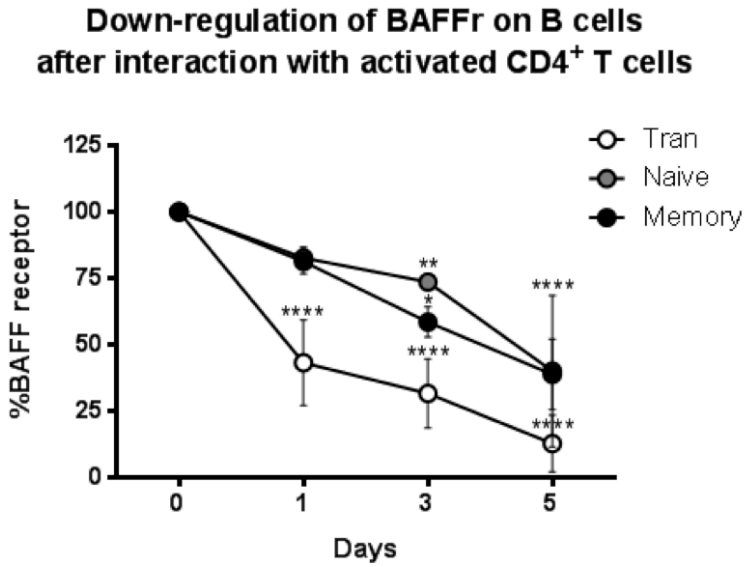
6.3.1 Memory, Naïve and Transitional B cell viability after five days of co-culture with $CD4^+$ T cells

After finding that Memory B cells induced proliferation, activation and cytokine production in low-activated autologous $CD4^+$ T cells, whereas Transitional B cells were incapable of inducing any activation, the properties of B cell subsets were studied in order to explain the differences observed in the $CD4^+$ T cell activation.

Cell apoptosis and viability were the first properties measured within the B cells. Down-regulation of BAFFr was used to measure apoptosis, and Live/Dead dye was used to measure viability as shown previously in Fig4 B and Fig4 A, respectively. Non-apoptotic cells were measured as $CD20^+BAFFr^+$ cells from the live population, after 1, 3 and 5 days of co-culture by surface staining (Fig28 A). Transitional B cells was the first population that experienced apoptosis and cell death, because at day 1, half of the cells significantly lost BAFFr expression (****$p<0.0001$), whereas 75% of Naïve and Memory B cells maintained their expression. By the third day of culture, all subsets experienced apoptosis and cell death, however, Transitional B cells were the population most affected by this (****$p<0.0001$). Finally, at day 5, Memory (****$p<0.0001$), Naïve (****$p<0.0001$) and Transitional B cells (****$p<0.0001$) presented the highest levels of cell death and apoptosis; Transitional B cells were more affected, compared to Memory ($p=0.0164$) and Naïve B cells ($p=0.0124$).

B cell viability at day five was measured in the large-scale experiment, and again, Memory B cells was the population with the highest survival rate, followed by Naïve and Transitional B cells (Fig28 B).

A



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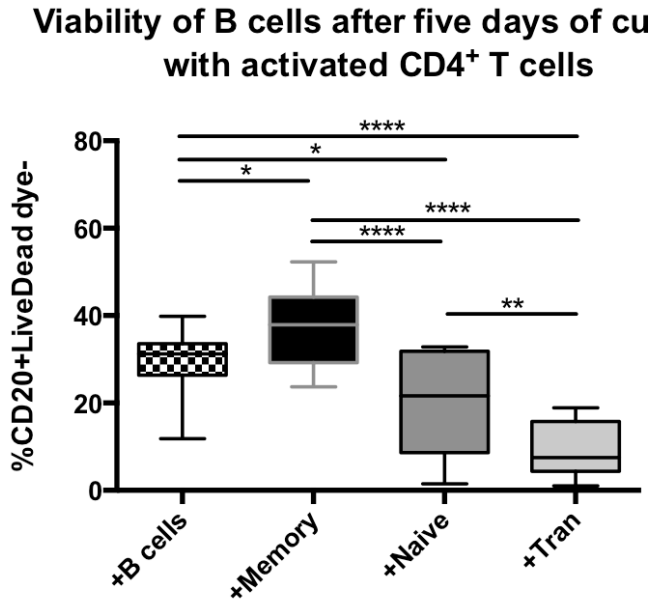


Figure 28: Measurement of viability and apoptosis in B cell subsets co-cultured with $CD4^+$ T cells.

B cell viability and apoptosis were measured in co-cultures of $0.5x10^6$ Memory, Naïve or Transitional B cells with $0.5x10^6$ anti-CD3-activated $CD4^+$ T cells at day 0, 1, 3 and 5 of culture; apoptotic live cells were identified as $CD20^+Live/Dead^-BAFFr^-$ (A). The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Tukey's multiple comparisons test. Viability of $0.5x10^6$ B cells, Memory, Naïve and Transitional B cells cultured with $0.5x10^6$ anti-CD3-activated $CD4^+$ T cells was measured after 5 days of co-culture; live cells were identified as $CD20^+Live/Dead^-$ (B). The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.3.2 Memory B cells exhibited higher levels of expression of CD86 and CD25 compared to Naïve and Transitional B cells, after five days of culture with $CD4^+$ T cells

CD86 and CD25 up-regulation on B cells induces T cell activation, *via* CD28 interaction, and cell survival, respectively. For this reason, expression of CD86 and CD25 was measured in B cell subsets after CD40 activation mediated by anti-CD3 activated $CD4^+$ T cells.

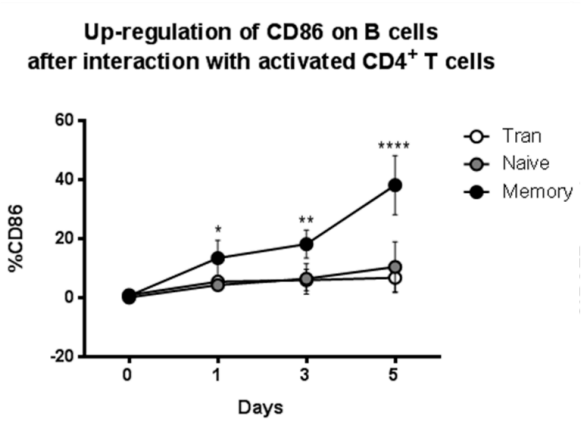
The same time course used to measure T cell proliferation and CD20 viability was used to measure CD86 expression at day 1, 3 and 5 of co-culture (Fig29 A). Memory B cells had the highest expression of CD86 at day 1 (*p=0.0160), 3 (**p=0.0017) and 5 (***p<0.0001) compared to the other subsets, exhibiting the highest expression at day 5. Naïve and Transitional B cells did not increase CD86 expression in this assay.

Expression of CD86 and CD25 was then measured at day 5 in the previously described large-scale experiment, and once again, an increase in CD86 expression was found in Memory B cells, compared to Naïve (*p=0.0489) and Transitional B cells (*p=0.0198) (Fig29 B). Regarding CD25 expression, as IL-2 was added in cultures, high expression of CD25 in all B cell subsets was predicted, however, Memory B cells exhibited the highest CD25 expression compared to Naïve (***p=0.0006) and Transitional B cell (**p=0.0046) (Fig29 C).

In conclusion, the differential CD86 expression observed in Memory B cells could explain the reason why these cells were inducing more activation on $CD4^+$ T cells. Low expression of this activation molecule in Transitional B cells corresponded with low T cell activation of these cells. CD25, on the other hand, could be an indirect signal of IL-2 capture, and therefore, an indicator of viability. This could imply that if Memory B cells have better survival compared to Naïve and Transitional (demonstrated by CD25 expression, viability and apoptosis results), they can activate $CD4^+$ T cells during a longer period of time before cell death.

These two main results, in isolation or together, provide an explanation of why Memory B cell were the main $CD4^+$ T cell activating cells and Transitional B cells were the lowest.

A



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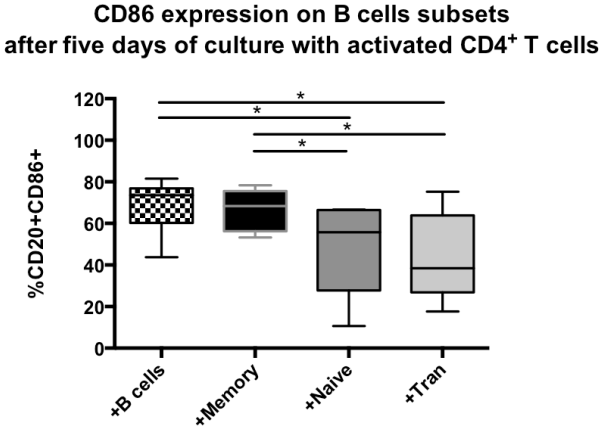

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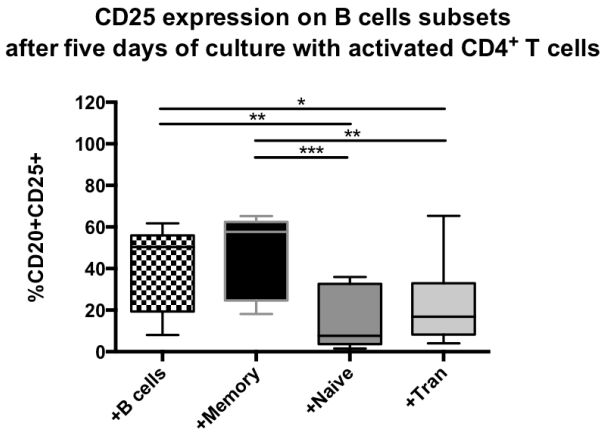

Figure 29: CD86 and CD25 expression in B cell subsets.

Expression of CD86 was measured in $0.5x10^6$ Memory, Naïve or Transitional B cells co-cultured with $0.5x10^6$ anti-CD3-activated $CD4^+$ T cells at day 0, 1, 3 and 5 of culture (A). The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Tukey's multiple comparisons test. Expression of CD86 (B) and CD25 (C) was measured in $0.5x10^6$ B cells, Memory, Naïve or Transitional B cells co-cultured with $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells after 5 days of culture. The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.3.3 Memory B cells produced higher levels of TNF-α compared to Naïve and Transitional B cells after five days of culture with $CD4^+$ T cells

Pro-inflammatory cytokine production was measured on B cell subsets to understand whether the presence of these cytokines was relevant for $CD4^+$ T cell activation. TNF-α and IFN-γ were measured, but only TNF-α was produced by B cells.

$CD20^+TNF\text{-}\alpha^+$ cells were measured in B cell subsets after 5 days of culture with activated $CD4^+$ T cells, and a significant increase of $CD20^+TNF\text{-}\alpha^+$ was observed mostly within the Memory B cells compared to Naïve B cells (****$p<0.0001$) and Transitional B cells (****$p<0.0001$) (Fig30).

In conclusion, Memory B cells were the main $CD4^+$ T cell-activating cells, as shown previously, and these cells were the main TNF-α-producing population as well. This cytokine is a well-known pro-inflammatory cytokine (Gaur *et al*. 2003), therefore it is possible to believe that this cytokine is actively participating in $CD4^+$ T cell activation, together with the other parameters described in the previous sections of this chapter.

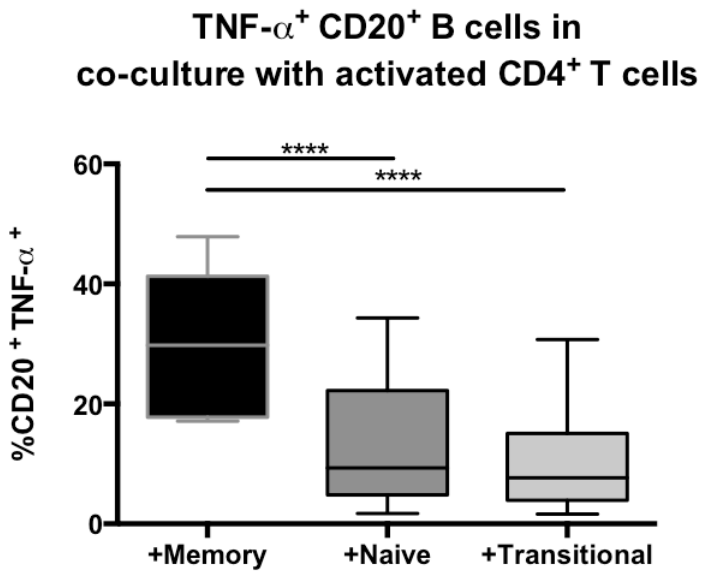

Figure 30: TNF-α expression in B cell subsets co-culture with $CD4^+$ T cells.

$CD20^+TNF\text{-}\alpha^+$ cells were identified in $0.5x10^6$ B cells, Memory, Naïve or Transitional B cells co-cultured with $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells after 5 days of culture. The statistical analysis used was RM-ANOVA test * $p<0.05$ with a Holm-Sidak's multiple comparisons test.

6.3.4 IL-10 production by B cell subsets and their effect in $CD4^+$ T cell proliferation

IL-10 has been shown to be a relevant anti-inflammatory cytokine in this study, since Blair *et al.* showed that Transitional B cells are capable of regulating Th1 responses through IL-10 production (Blair *et al*. 2010). CD40-CD40L interaction induced IL-10 production specifically by Transitional B cells, as was shown in Fig11 B and C. Because of these two main results, production of IL-10 was studied by B cell subsets after CD40 activation using intracellular staining, and then, the role of IL-10 in $CD4^+$ T cell proliferation was analysed by using a neutralising IL-10 receptor antibody.

In the same large-scale experiment performed previously, IL-10 production was measured in Memory, Naïve and Transitional B cells after 3 and 5 days of culture with anti-CD3 activated $CD4^+$ T cells, using intracellular staining. Examples of $CD20^+IL\text{-}10^+$ B cells non-activated and activated with $CD4^+$ T cells after 3 (Fig31 A) and 5 (Fig31 B) days of co-culture are presented in dot plots.

$CD20^+IL\text{-}10^+$ Memory, Naïve and Transitional B cells from all the experiments performed were plotted at day 3 (Fig31 C) and 5 (Fig31 D) after co-culture with anti-CD3 (1ug/ml) activated $CD4^+$ T cells. Significant differences between B cell subsets were observed at day 3, but not at day 5, and the percentages of $CD20^+IL\text{-}10^+$ Transitional B cells were significantly higher compared to Memory B cells (*p=0.0282). Percentages of $CD20^+IL\text{-}10^+$ Naïve B cells were also significantly higher compared to Memory B cells (*p=0.0282), but no difference between Naïve and Transitional were observed at day 3. In terms of day 5, no differences were observed between B cell subsets, and percentages of all $CD20^+IL\text{-}10^+$ B cell subsets were lower compared to day 3.

Finally, the effect of IL-10 on $CD4^+$ T cell proliferation was studied (Fig31 E). In order to evaluate the effect of IL-10 produced by B cell subsets on $CD4^+$ T cell proliferation, a neutralising IL-10 receptor antibody was used in the $CD4^+$ T cell-B cell co-cultures. Proliferation was measured with a Violet Cell Trace dye in $CD4^+$ T cell, and results were presented as peaks of proliferation cycles using histograms. The blocking effect of IL-10 moderately increased proliferation in $CD4^+$ T cells cultured alone, did not induce any difference in Memory B cells, and induced a higher

proliferation in $CD4^+$ T cells cultured with Naïve and Transitional B cells. Although an increased proliferation was observed in $CD4^+$ T cells cultured with Naïve and Transitional B cells, only $CD4^+$ T cells co-cultured with Transitional B cells reached statistical significance (Fig31 F).

In this last set of experiments, a differential release of IL-10 was observed between day 3 and 5 of culture, with IL-10 present at day 3 but absent at day 5. Looking at $CD4^+$ T cell proliferation, proliferation between the third and the fifth day of culture could be seen, but was very low prior to these days (Fig25 B). Together, these two sets of results suggest that IL-10 is participating in the control of the T cell proliferation when Naïve and Transitional B cells were co-cultured with T cells.

Interestingly, increments of T cell proliferation was observed when the IL-10 neutralising antibody was added into $CD4^+$ T cells cultured without B cells; this may be explained because of the effect of regulatory $CD4^+$ T cells present in the $CD4^+$ T population. Regulatory T cells secrete IL-10 (Barrat *et al*. 2002; Taylor *et al*. 2006), therefore if this pathway was blocked in $CD4^+$ T cell cultures, $CD4^+CD25^-$ T cells should proliferate more, as IL-10 cannot be up taken by these cells. In contrast, differences were observed when IL-10 was neutralised in co-cultures with Memory B cells, although, Memory B cells were the lowest IL-10 producing cells after CD40 activation.

Finally, T cell proliferation induced by Naïve and Transitional B cells was severely affected by IL-10 blocking. Proliferation was significantly increased in co-cultures with Transitional B cells, suggesting that IL-10 production by Transitional B cells was important in the control of the proliferation. Activation markers and pro-inflammatory cytokine secretion were also increased in $CD4^+$ T cell – Transitional B cells co-cultures when anti-IL-10 receptor antibody was present in culture (data not shown). All these data suggest that IL-10, produced by Transitional B cells, is regulating T cell activation and proliferation.

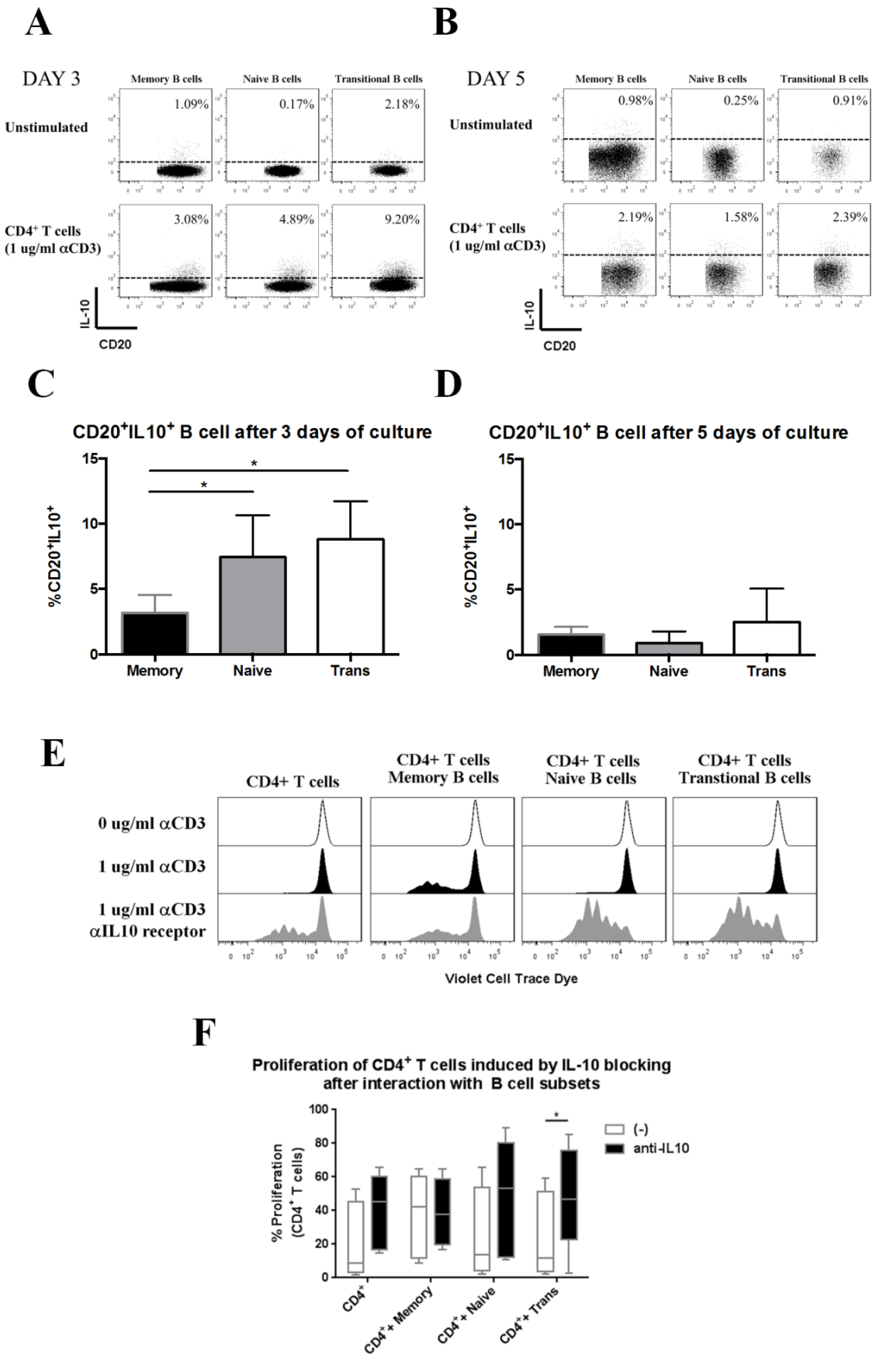
A
B
DAY 3
Memory B cells
Naive B cells
Transitional B cells
Unstimulated
1.09%
0.17%
2.18%
CD4+ T cells
(1 ug/ml αCD3)
3.08%
4.89%
9.20%
IL-10
CD20
DAY 5
Memory B cells
Naive B cells
Transitional B cells
Unstimulated
0.98%
0.25%
0.91%
CD4+ T cells
(1 ug/ml αCD3)
2.19%
1.58%
2.39%
IL-10
CD20
C
D
CD20+IL10+ B cell after 3 days of culture
%CD20+IL10+
15
10
5
0
*
*
Memory
Naive
Trans
CD20+IL10+ B cell after 5 days of culture
%CD20+IL10+
15
10
5
0
Memory
Naive
Trans
E
CD4+ T cells
CD4+ T cells
Memory B cells
CD4+ T cells
Naive B cells
CD4+ T cells
Transtional B cells
0 ug/ml αCD3
1 ug/ml αCD3
1 ug/ml αCD3
αIL10 receptor
Violet Cell Trace Dye
F
Proliferation of CD4+ T cells induced by IL-10 blocking
after interaction with B cell subsets
% Proliferation
(CD4+ T cells)
100
80
60
40
20
0
*
(-)
anti-IL10
CD4+
CD4+ + Memory
CD4+ + Naive
CD4+ + Trans

Figure 31: IL-10 expression on B cell subsets after 3 and 5 days of co-culture with $CD4^+$ T cells.

Dot-plots examples of $CD20^+IL\text{-}10^+$ B cells found co-cultures of $0.5x10^6$ Memory, Naïve or Transitional B cells with $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells after 3 (A) and 5 (B) days of culture. $CD20^+IL\text{-}10^+$ cells were identified in $0.5x10^6$ Memory, Naïve or Transitional B cells co-cultured with $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells after 3 (C) and 5 (D) days of culture. Friedman test with a Dunn's multiple comparisons test was used; * $p<0.05$ was considered significant. Histograms of T cell proliferation (E) and total percentages of T cell proliferation (F) of $1.0x10^6$ anti-CD3-activated $CD4^+$ T cells co-cultured with or without $0.5x10^6$ Memory, Naïve or Transitional B cells, and with and without blocking anti-IL-10 receptor (0.1ng/ml), after 5 days of culture. Two-way RM ANOVA test with a Sidak's multiple comparisons test was used; * $p<0.05$ was considered significant.

6.3.5 Expression of IL-10 receptor on B cell subsets

Finally, the expression of IL-10 receptor on B cell subsets was evaluated by surface staining in order to identify whether B cells could be consuming IL-10.

Sorted Memory, Naïve and Transitional B cells were stained with an anti-IL-10 receptor antibody; examples of the staining are presented in dot-plots of $CD20^{+}$IL-10-$receptor^{+}$ cells from Memory, Naïve and Transitional B cell samples (Fig32 A). Overall, the expression of the IL-10 receptor was significantly increased on Transitional B cells compared to Naïve B cells before culture (Fig32 B).

In summary, these results showed an increased expression of the IL-10 receptor in the Transitional population, compared to Memory and Naïve B cells. This may indicate that IL-10 could be playing an important role in the Transitional B cell function, perhaps either as a survival signals, as an activation signal, or as a positive signal for IL-10 positive feedback. This could also explain the conflicting results obtained when IL-10 was measured in supernatants, possibly because Transitional B cells were not only secreting this cytokine, they were also consuming it. Why Transitional B cells exhibited a high expression of the IL-10 receptor, or what is the role of IL-10 in these cells will require more experiments and future work.

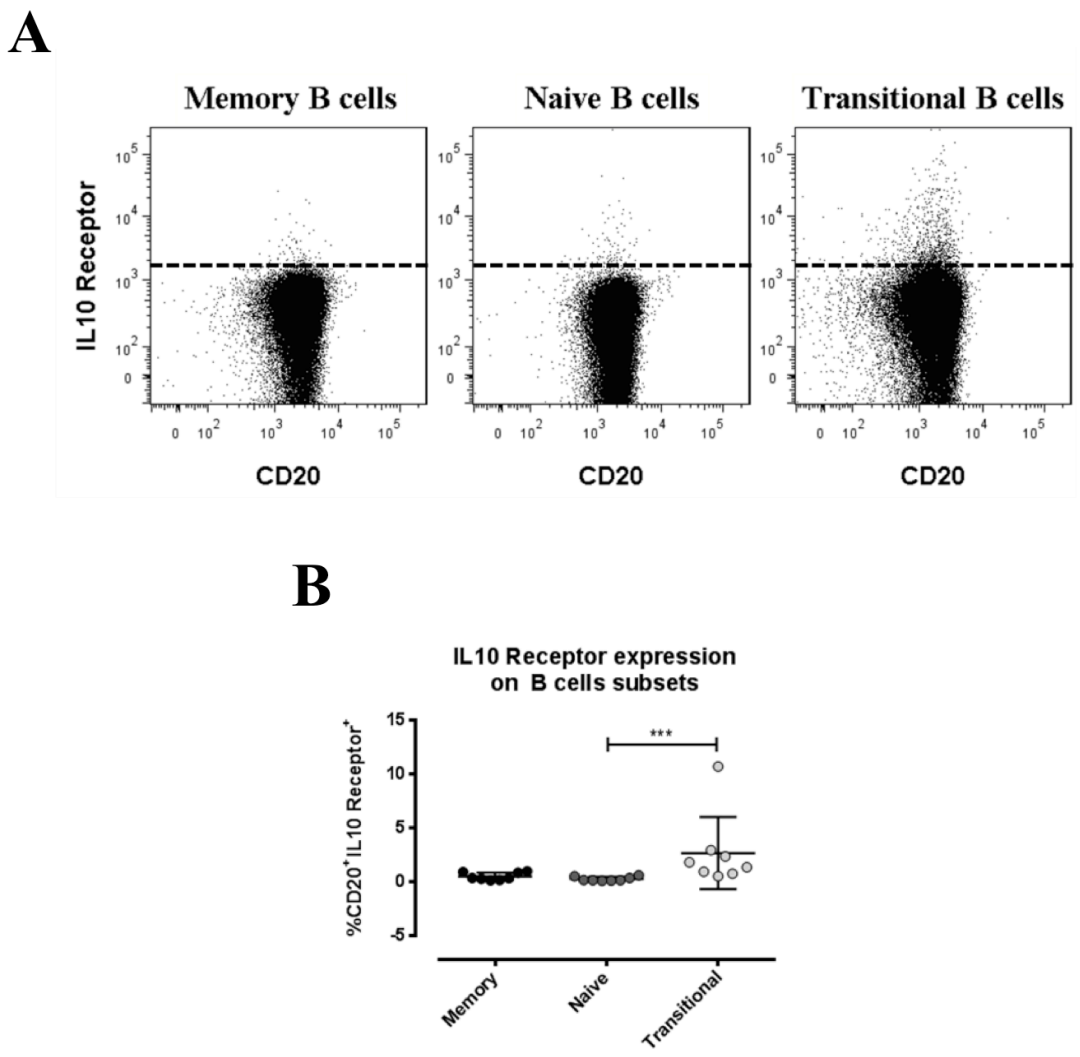

Figure 32: Expression of IL-10 receptor in B cell subsets.

Expression of IL-10 receptor was observed in Memory, Naïve and Transitional B cells (A). $CD20^{+}$IL-10 $receptor^{+}$ B cells were measured in $0.5x10^{6}$ Memory, Naïve and Transitional B cells samples by surface staining (B). Friedman test with a Dunn's multiple comparisons test was used; * $p<0.05$ was considered significant.

6.4 Discussion.

6.4.1 Overview

The cognate interaction between B and T cells through MHC-class II-TCR is crucial for both lymphocytes as it can lead to T and B cell activation, cytokine production, proliferation and antibody production (O'Rourke *et al*. 1997). Another interaction between the same two cells has been shown to be important in the immune regulation and anti-inflammatory responses in humans; namely CD40/CD40L interaction in the absence of BCR-specific recognition. Blair at al. described that CD40-activated $CD19^{+}CD24^{hi}CD38^{hi}$ B cells were capable of inhibiting the Th1 response, *in vitro*. They demonstrated that this interaction induced a B cell suppressive response mediated by IL-10 production and CD80 and CD86 expression (Blair *et al*. 2010). This chapter was designed to evaluate T cell activation induced by CD40 activated B cells *in vitr*o, to then apply this principles in the context of transplantation tolerance.

6.4.2 T cell activation and proliferation

The CD40-CD40L interaction was studied to evaluate the immune responses induced by B cell subsets in $CD4^{+}$ T cells and the first parameter analysed in this chapter was the T cell proliferation. It is well described that CD3 activation induces T cell proliferation (Meuer *et al*. 1984); it is also known that CD28 enhances this proliferation when it is activated at the same time as CD3 (Azuma *et al*. 1992); besides proliferation, these two signals can induce CD40L up-regulation, and consequently, B cell activation (Srahna *et al*. 2001). It was found that activation with anti-CD3/CD28 antibodies or with high concentrations of anti-CD3 antibody, not only induced up-regulation of CD40L, but also induced high levels of $CD4^{+}$ T cell activation and proliferation. These effects did not seem to be mediated by the B cell subsets. Low concentrations of anti-CD3 antibody were then used to determine differences between the B cell subsets. These results imply that in the context of transplantation, B cells can regulate or mediate T cell activation in a CD40-CD40L specific manner, only when T cell activation is low. Then, unactivated T cells do not up-regulate CD40L and therefore, do not activate B cell subsets, whilst highly activated T cells do not respond to B cell mediation.

6.4.3 IL-10 production by Transitional B cells

Claudia Mauri's group in 2010 demonstrated for the first time in human cells, that only Transitional B cells had the ability to suppress the Th1 response after CD40 activation *via* IL-10 production (Blair *et al*. 2010). Following on from these findings, the Th1 response in B-T cell co-cultures was measured in this thesis to evaluate the capacity of the B cells to induce T cell activation and cytokine production. These results showed that Transitional B cells did not induce activation nor cytokine production, whereas Blair *et al*. showed that Transitional B cells inhibited IFN-γ and TNF-α secretion by T cells (Blair *et al*. 2010). In summary, two different experimental designs gave the same message; only Transitional B cells regulate the pro-inflammatory response generated by activated T cells.

Cytokine production was measured in B cells during co-culture with $CD4^+$ T cells. Memory B cells were the highest TNF-α-producing cells within total B cells. It was shown that TNF-α can induce T cell activation, proliferation and apoptosis (Gaur *et al*. 2003), depending on the surrounding signals, therefore, Memory B cells may be inducing T cell activation through TNF-α secretion in this system. On the other hand, the anti-inflammatory response was regulated by IL-10, a cytokine that inhibits human T cell proliferation and activation (Taga *et al*. 1992; Sieg *et al*. 1996; Ebert 2000; Lemoine *et al*. 2011). This cytokine was identified after 72 hours of activation, predominantly in the Transitional B cells, secondly in Naïve B cells and poorly in the Memory B cell population; interestingly, the signal was completely lost afterwards. Because Naïve and Transitional B cells were the main IL-10 producing cells compared to Memory B cells, IL-10 was associated with the regulatory control of $CD4^+$ T cell activation. Interestingly, IL-10 secretion during the first three days of culture was consistent with the low $CD4^+$ T cell proliferation rate observed. After day three, B cells stopped the secretion of IL-10 the same time that proliferation started to increase.

In order to evaluate any effects of B cell-induced IL-10, an anti-IL-10 receptor was used in co-cultures to block this signalling pathway in cells that were consuming the cytokine. Interestingly, increments in $CD4^+$ T cell proliferation were observed in co-cultures with Naïve and Transitional B cells when the anti-IL-10 receptor was added. In addition, a moderate increment of proliferation was observed in the $CD4^+$ T cells alone, after the addition of the anti-IL-10 receptor. As it has been reported that regulatory T cells (Barrat *et al*. 2002; Taylor *et al*. 2006) and B cells (Bouaziz *et al*. 2010) regulate

proliferation through IL-10 secretion, the increment in the proliferation observed in the $CD4^+$ T cell condition must be due of the blocking effect of regulatory T cells, as they were not excluded from the system. Proliferation was not affected in co-cultures with Memory B cells when anti-IL-10 receptor was added; in fact the variation between samples was minimal.

When the IL-10 pathway was blocked, Transitional and Naïve B cells induced a higher T cell proliferation, activation and cytokine production (data not shown) compared to Memory B cells, this suggests that IL-10 produced by these two populations is able to regulate T cell proliferation in an IL-10-dependent manner. These results imply either an immune regulation through a direct mechanism, in which IL-10 produced by B cells is directly regulating T cell proliferation; or an indirect mechanism in which IL-10 produced by B cells is regulating a T cell subset (within total $CD4^+$ T cells) that in turn regulates proliferation; or a combination of both mechanisms. Flores-Borja *et al*. reported that Transitional B cells convert effector T cells into Tregs partially *via* the release of IL-10 (Flores-Borja *et al*. 2013). In this chapter IL-10 secretion and FoxP3 expression were measured in T cells co-cultured with different B cell subsets but no significant differences in the expression of this Treg marker could be found (data not shown). However this thesis does not disagree with Flores-Borja's study since results from the $CD4^+$ T cells cultured only with the blocking IL-10 antibody suggest that the regulatory properties of a T cell subset within total $CD4^+$ T cells was negatively affected. Roncarollo's group showed that $CD49b^+LAG\text{-}3^+$ Tr1 cells secrete large amounts of IL-10 (Gagliani *et al*. 2013), this population may also be affected by the IL-10 blocking antibody and it would be interesting to evaluate the interaction between Transitional B cells and these cells.

In summary the main idea of this chapter was to demonstrate the differences between B cell subsets in the induction of T cell proliferation, activation and cytokine production. From all the results generated in these co-culture assays, it can be concluded that Transitional B cells are not efficient activator cells, as they do not induce T cell proliferation, activation, or pro-inflammatory cytokine production. They produced IL-10 after CD40 activation, mainly at the beginning of the culture, and during this time, they controlled T cell proliferation *via* this cytokine release. On the contrary, Memory B cells exhibited better viability, higher CD86 expression, high TNF-α production and low IL-10 secretion. Because these cells had all the features required to be a proper T

cell activating-cell, it is not surprising that they induced more proliferation, activation and cytokine production than the other subsets.

7 Discussion

Kidney transplant recipients that developed spontaneous tolerance exhibited maintained percentages and numbers of total B cells and Transitional B cells in circulation (Newell *et al*. 2010; Pallier *et al*. 2010; Sagoo *et al*. 2010; Silva *et al*. 2012). B cells have been traditionally linked to antibody production (Hidalgo *et al*. 2009), and hence, a detrimental graft function (Kimball *et al*. 2011; Loupy *et al*. 2012; Mohan *et al*. 2012; Page *et al*. 2012). Thus, a protective or regulatory function by B cells has been unexpected in transplantation. In order to clarify the role of this population in this clinical field, different groups have use Rituximab to reduce acute and/or chronic rejection *via* inhibition of B cell participation in the immune response, however results are contradictory (Becker *et al*. 2004; Faguer *et al*. 2007; Clatworthy *et al*. 2009), and the role of the B cells in transplantation still remains inconclusive. The existence of different B cell subsets inducing or participating in different immune responses may explain these conflicting results (Sims *et al*. 2005; Palanichamy *et al*. 2009).

Transitional B cells have been proposed as the main regulatory population within human B cells as they are able to regulate the pro-inflammatory Th1 response (Blair *et al*. 2010) and to induce regulatory T cells (Flores-Borja *et al*. 2013) *via* IL-10 production. However, there are several aspects that must be considered when these cells are proposed as a regulatory source in transplantation tolerance. Transitional B cells are about 5-10% of B cells and 1% of total PBMCs (Bouaziz *et al*. 2010). They can be found mainly in the bone marrow and peripheral blood (Sims *et al*. 2005), rather than in lymphoid organs (Cuss *et al*. 2006). They are in a transitory state as they do not maintain their phenotype over time, and they easily undergo to apoptosis and cell death after activation (Sims *et al*. 2005). How is it then possible that a small and fragile population is regulating or modulating tolerance in kidney transplant recipients? Three different mechanisms are described in this thesis to try to understand the role of Transitional B cells in this particular clinical state.

This study proposes that Transitional B cells contribute to tolerance in kidney transplant recipients by IL-10 production, low levels of BCR activation and absence of donor-specific responses. These three mechanisms are not necessarily independent of each other, and they depend on the type of activation received by the B cells.

In the first mechanism this study proposes that in tolerant recipients, low activated T cells in circulation are able to activate Transitional B cells *via* CD40-CD40L interaction in a non-antigen specific manner. As a consequence of this activation, Transitional B cells secrete IL-10 that inhibits cell proliferation only in low activated T cells. This inhibition possibly reduces the activation of the T cells in tolerant patients; therefore T cell activation will be lower in these, compared to patients with low numbers of Transitional B cells within the total B cell population.

In the second mechanism, the signalling pathway triggered after BCR activation is decreased specifically in all B cell subsets from tolerant recipients, preventing or diminishing B cell activation and Plasma cell differentiation after antigen encounter. Also it is known that Memory B cells produce LT and TNF-α after BCR/CD40 stimulation, whereas Naïve and Transitional B cells secreted mainly IL-10 after CD40 stimulation alone (Duddy *et al*. 2004; Duddy *et al*. 2007). Therefore, if B cells exhibited a defect in BCR activation, Memory B cells will not produce LT and TNF-α, and Naïve and Transitional B cells will mainly produce IL-10, not only after CD40 activation, but also in combination with BCR stimulation.

In the last mechanism donor-specific responses mediated by the different B cells subsets were measured in kidney transplant recipients. Results showed that all B cells subsets from tolerant recipients failed to induce a Th1 response. These results could be explained because: 1) all B cells from tolerant recipients exhibited a defect in the BCR signaling pathway; 2) B cells from tolerant recipients did not recognised and/or internalise donor-antigens; 3) B cells from tolerant recipients did not induce any response after donor-antigens presentation; 4) T cells from tolerant recipients did not respond after donor-antigen presentation, specifically by B cells; and/or 5) differences between antigens from tolerant recipients and their respective donors are not sufficient (in numbers or antigenicity) to induce a B or T cell response. When the same mechanism was studied in chronic rejector, results revealed that only Transitional B cells failed to induce a Th1 response compared to Naïve and Memory B cells, demonstrating a specific defect by Transitional B cells in donor-specific presentation.

In conclusion this thesis proposes that these three mechanisms, together or independent from each other, are contributing to kidney transplantation tolerance. Although the presence of Transitional B cells may be beneficial to regulate donor-

specific responses and to inhibit T cell activation, other clinical and immunological parameters need to be considered to establish tolerance in kidney transplant recipients.

Major Findings

8.1 Characterisation of Transitional B cells in kidney transplant patients.

1. Transitional B cells were maintained in tolerant recipients.
2. Higher percentages of Transitional B cells were found in peripheral blood rather than spleen or lymph nodes.
3. Transitional B cells exhibited a higher density of CD20, but not CD19, compared to other B cell subsets.
4. B cells from stable and chronic rejector patients exhibited a lower BAFFr expression which implies a lower B cell viability compared to tolerant and healthy controls.
5. CD25 and CD86 were up-regulated in B cells after CD40L activation, but only CD25 expression was significantly increased in B cells from tolerant recipients compared to chronic rejector.
6. TLR-9 was up-regulated in B cells after CpG activation in all patient groups except chronic rejector.
7. CpG induced IL-10 production mainly by Memory B cells, while CD40L induced IL-10 mainly by Transitional B cells.
8. B cells from tolerant recipients produced higher levels of IL-10 compared to B cells from chronic rejector after CD40L activation. This difference was due to different percentages of Transitional B cells between these two groups.
9. B cells from tolerant recipients produced higher levels of IL-10 compared to B cells from chronic rejector after CpG activation. Despite that, non-B cells from chronic rejector also produced IL-10 as evidenced in ELISA results.
10. Patients who lost tolerance exhibited a decrease in the percentages of Transitional B cells, despite total B cells remained unchanged. B cells from these patients did not secrete IL-10 after CD40 activation.

8.2 Donor-specific responses in kidney transplant patients.

1. B cell activation *via* the BCR was lower in B cells from tolerant recipients, compared to B cells from healthy controls. This suggests that B cells from tolerant recipients showed an anergic or non-responsive state.
2. BCR activation was higher in Memory B cells compared to Transitional B cells from healthy controls. This pattern was not observed in kidney transplant recipients, except chronic rejector.
3. Donor-specific proteins were predominantly recognised by B cells from chronic rejector. After antigen processing and presentation, B cells from tolerant recipients failed to induce a Th1 response. On the other hand, almost all B cell subsets from stable and chronic rejector patients induced IFN-γ production by $CD4^+$ T cells. Only Transitional B cells from chronic rejector failed to induce a Th1 response. In terms of IL-10 effects, no participation of this cytokine was observed in donor-antigen responses in tolerant recipients.
4. HLA specificities were equally distributed in DSAs from kidney transplant recipients. Chronic rejector was the group with the most positive patients for DSAs, suggesting a direct relationship between DSA and rejection, but because three tolerant recipients also presented DSAs, the presence of these antibodies in serum does not necessarily correlate with graft rejection.

8.3 B cell subsets and $CD4^+$ T cell interaction.

1. Transitional B cells did not induce $CD4^+$ T cell proliferation, activation and pro-inflammatory cytokine production compared to Memory B cells.
2. Transitional B cells exhibited the lowest viability rates, compared to Naïve and Memory B cells.
3. Memory B cells expressed high levels of CD86, CD25 and TNF-α after $CD4^+$ T cell activation, compared to Transitional B cells.
4. IL-10 was secreted by all B cell subsets, but mostly by Transitional B cells, after 3 days of $CD4^+$ T cell activation; the secretion of this cytokine was lost at day 5. IL-10 was controlling proliferation in T cells co-cultured with Naïve and Transitional B cells, but not in T cells co-cultured Memory B cells.

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Appendix

Genes differentially expressed in kidney transplant patients and healthy controls.

A genetic B cell signature associated to renal transplant tolerance was found in humans in three independent cohorts (Brouard *et al*. 2007; Newell *et al*. 2010; Pallier *et al*. 2010; Sagoo *et al*. 2010). These studies identified several transcriptional biomarkers in peripheral blood related to B cells; *MS4A1*, the CD20-encoding gene, *CD79B*, a member of the B cell co-receptor, *FCRL1* and *FCRL 2*, two Fc receptor-like proteins and genes related with the immunoglobulin conformation as *IGKV4-1*, *IGLL1*, and *IGKV1D-13*. The genetic B cell signature found in all these studies revealed at the same time, an augmented number of B cells in tolerant recipient compared to stables and chronic rejector; these suggest that presence of genes related to B cells, may be a potential biomarker of transplantation tolerance.

In this thesis, B cell genes were evaluated in isolated B cells, to identify any relevant gene that could help to understand the mechanism or the relationship between B cells and transplantation tolerance. The results from the microarray used in this study presented some technical problems due to the low number of B cells in some patients, but they were solved with the pre-amplification of critical samples. Five patient samples per groups were included from tolerant, chronic rejector, healthy controls and stable patients. Results did not reveal any significant p value due to a low number of samples, but an interesting tendency was observed in some relevant B genes (Fig33). Thus this section has been added as an appendix.

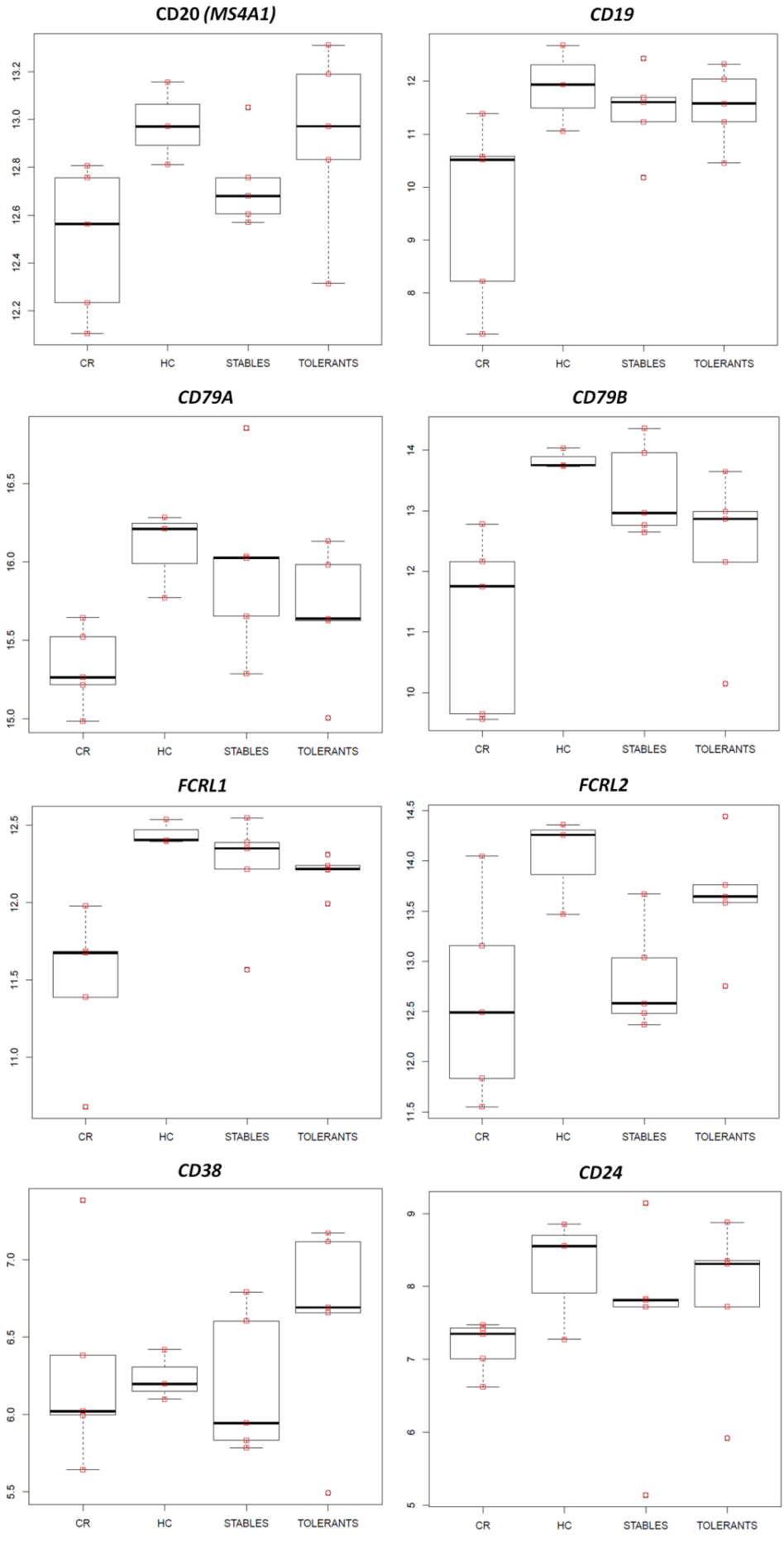
CD20-encoding gene, exhibited a higher expression in tolerant patients compared to stable, and even more compared to chronic rejector; this finding was interesting as Transitional B cells were shown to express high levels of this protein in chapter 4 (Fig7 A), therefore the gene expression in periphery was consistent with protein levels in the cell surface.

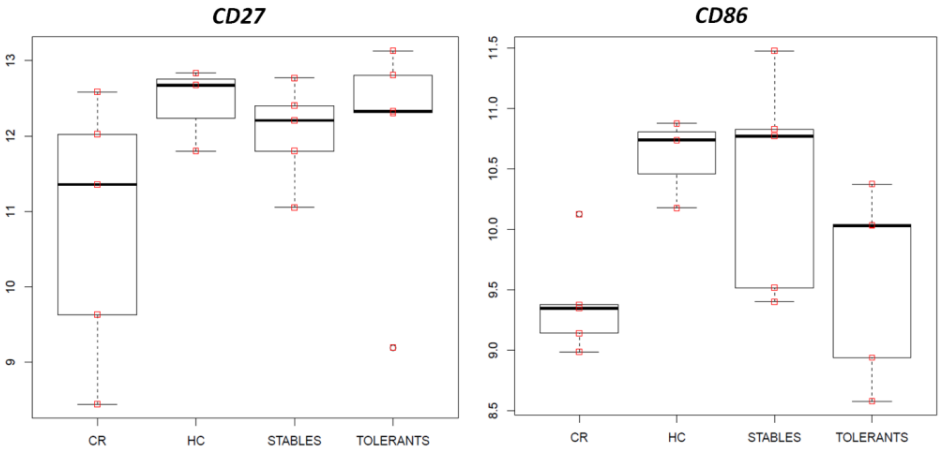
CD19, CD79A, CD79B, FCRL1, FCRL2 and *CD27* shared a similar pattern than CD20, in which chronic rejector always exhibited the lowest expression of these genes compared to other groups.

CD38 and *CD24* genes were increased in tolerant B cell samples; this result can be explained by the fact that tolerant patient exhibited a higher percentage of $CD24^{hi}CD38^{hi}$ Transitional B cells (Fig3 D).

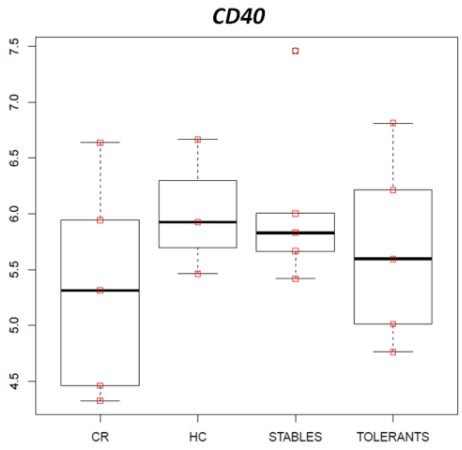
Interestingly, *CD86* and *ERK*, two genes related with post-activation signals, were decreased in both tolerant and chronic rejector. These results were consistent with ERK-p experiments from the second chapter, in which B cells from tolerant patients failed to phosphorylate ERK after BCR activation. They were also consistent with CD86 expression, as no differences in neither non-activated nor activated samples between patients groups were observed in the first chapter. *CD40* did not exhibit any difference in both, gene or protein expression. No clear difference were observed regarding TLR-9 expression (cells were not CpG-activated). Finally, *BLK* and *BLNK* exhibited the same pattern than phenotypic markers.

In summary, gene expression from isolated B cells was consistent with protein expression found in different kidney transplant recipients. Unfortunately, because of the low number of patients per group, no statistical significance was found in the microarrays; more patients are required to complete this chapter.


CD20 (MS4A1)
CD19
CD79A
CD79B
FCRL1
FCRL2
CD38
CD24
CR
HC
STABLES
TOLERANTS


CD27
13
12
11
10
9
CR
HC
STABLES
TOLERANTS

CD86
11.5
11.0
10.5
10.0
9.5
9.0
8.5
CR
HC
STABLES
TOLERANTS


CD40
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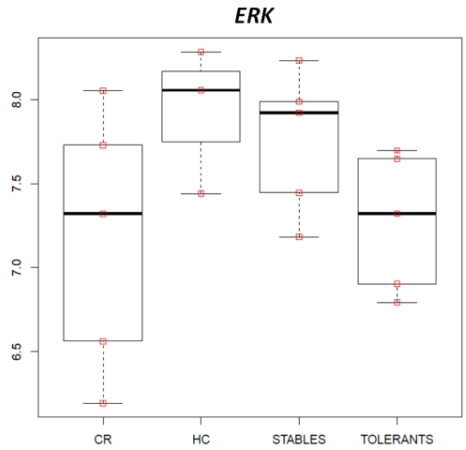
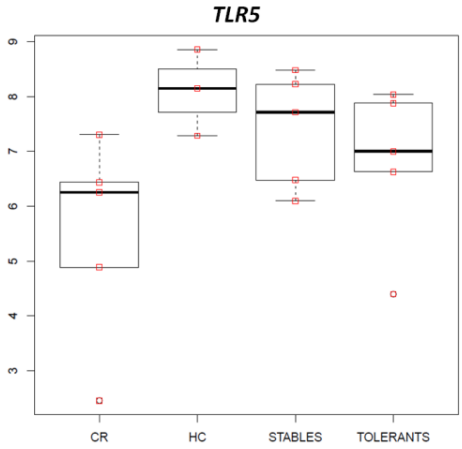
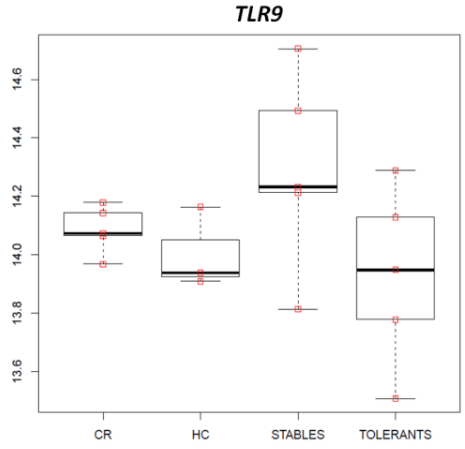
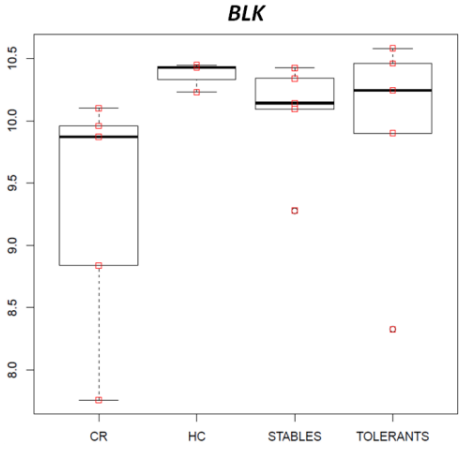
7.0
6.5
6.0
5.5
5.0
4.5
CR
HC
STABLES
TOLERANTS


ERK
8.0
7.5
7.0
6.5
CR
HC
STABLES
TOLERANTS


TLR5
9
8
7
6
5
4
3
CR
HC
STABLES
TOLERANTS


TLR9
14.6
14.4
14.2
14.0
13.8
13.6
CR
HC
STABLES
TOLERANTS


BLK
10.5
10.0
9.5
9.0
8.5
8.0
CR
HC
STABLES
TOLERANTS

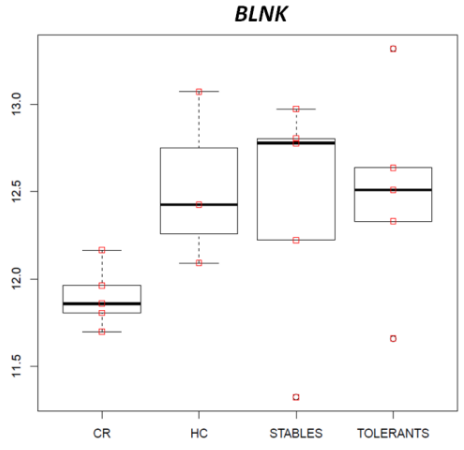

BLNK
13.0
12.5
12.0
11.5
CR
HC
STABLES
TOLERANTS

Figure 33: B cell-related genes expressed in healthy controls and kidney transplant patients.

Box-plots of *MS4A1, CD19, CD79A, CD79B, FCRL1, FCRL2, CD38*, *CD24, CD27, CD86*, *CD40, ERK, TLR5, TLR9*, *BLK* and *BLNK* expression in isolated B cells from healthy control (HC), tolerant, stable and chronic rejector (CR). Graphs were plotted using SPSS Statistics.

Discussion

CD20, CD79b, FcRL1 and FcRL2 are the proteins encoded by the genes *MS4A1, CD79B, FCRL1 and FCRL2,* respectively. These genes were associated to kidney transplantation tolerance (Brouard *et al*. 2007; Newell *et al*. 2010; Sagoo *et al*. 2010).

CD20 was the first differentiation marker identified in human B cells, and *MS4A1* is the name of the gene that encodes this protein. This was the fifth gene differentially high expressed in tolerant patients from the IOT study (Sagoo *et al*. 2010), was also identified in the ITN study (Newell *et al*. 2010) and Brouard's study (Brouard *et al*. 2007). Moreover, only the CD20 transcript from urine sediments was significantly higher in tolerant patients compared to patients with stable function or healthy controls (Newell *et al*. 2010). CD20 was also differentially expressed between B cell subsets, in which Transitional B cells expressed higher levels of CD20 compared to Naïve and Memory B cells (Cuss *et al*. 2006). Because CD20 was highly expressed in tolerant recipients, and especially in Transitional B cells, a potential role as a biomarker can be attributed to this protein.

The functional role of the CD20 is not well defined in the literature; therefore, it is difficult to establish a mechanistic relation between CD20 and tolerance. The only evidence obtained from a reported case of a patient that naturally did not express CD20 on B cells, demonstrated that this protein was important in the generation of T cell–independent (TI) antibody responses (Kuijpers *et al*. 2010); however, no participation was observed in B cell proliferation or activation. But because biomarkers are not necessary required to have a specific function, CD20 can still be considered to detect tolerance. This marker could be a direct indication of an increased number of B cells in the patient, but this seems unlikely as *CD19 gene* was much lower in the significance scale in the IOT microarray (Sagoo *et al*. 2010).

A new microarray was performed in our group of kidney transplant patient, this time isolated B cells were used, instead of whole blood used in previous reports. The idea was to identify any specific role of B cell-related genes in tolerance or rejection. Here, *MS4A1* and *CD19* gene expression was fairly similar in all patient groups. Healthy controls, stable and tolerant recipients exhibited a similar expression of *CD19*, but only healthy controls and tolerant recipient exhibited a similar *MS4A1* expression,

while in stable it remained low and even closer to chronic rejector that exhibited the lowest expression in both genes.

CD79 is the signalling component of the BCR and its signal is triggered after the BCR cross-linking. This interaction can induce apoptosis or, in the presence of a rescue signals from T cells, can drive cell activation and division (Niiro *et al*. 2002). *CD79B* was the first, and *CD79A* was the eighteenth gene differentially expressed in tolerant patients in the IOT study (Sagoo *et al*. 2010). CD79B expression was measured by flow cytometry but no differences were found between patient groups (data not shown). Regarding the microarray, *CD79A* and *CD79B* exhibited the same expression in all groups, except chronic rejector; again they exhibited the lowest expression in both genes.

FcRL1 and FcRL2 are transmembrane glycoproteins from a large family of Fc receptor-like (FCRL) molecules. Although they share a common ancestor with the classical Ig-binding Fc receptors (FCR), these proteins do not bind Igs, therefore cannot be considered Fc receptors (Davis 2007). FCRL1–5 are all expressed in B cells; however, the distribution of these proteins is variable depending on the subpopulation (Davis 2007). FcRL1 ligation enhances BCR-induced Ca^{2+} mobilization and cell proliferation, suggesting a potential role as an activating protein of the B cell co-receptor (Leu *et al*. 2005). FcRL1 and FcRL2 were two proteins encoded by genes differentially expressed in tolerant recipients found in the IOT study and in the INT study (Newell *et al*. 2010). Both proteins are highly expressed on B cells, and their transcription is predominantly high in B cells from the follicular mantle zone, mainly Naïve B cells (Davis 2007). Preliminary results from the microarray shown the presence of FcRL1 on B cells, but not on $CD14^+$ cells (data not shown), corresponding with published data that reported that FcRL1 was found on all of circulating B cells, but not on T cells, NKs, monocyte/macrophages, granulocytes, and platelets (Leu *et al*. 2005). Despite the fact that literature had only reported FcRL2 on $CD20^+$ B cells, and mainly in human $CD27^+$ Memory B cells (Davis 2007), FcRL2 protein expression was found in this study in both populations, $CD20^+$ and $CD14^+$ cells (data not shown). Whereas *FCRL1*, as CD19, *CD79A* and *CD79B,* exhibited a high expression in all group samples except in chronic rejector, *FCRL2, as CD20,* exhibited a lower expression in stable patient, and even lower in chronic rejector compared to healthy

controls and tolerant recipients. Despite all this data, the relation between both proteins and tolerance is still unknown.

CD27, CD24 and *CD38* are B cell membrane proteins and their differential expression allowed identifying Memory, Naïve and Transitional by surface staining. Chronic rejector samples had reduced expression in *CD27*, but no difference was observed between healthy controls, stable and tolerant patients. *CD24* and *CD38* were at the limit of detection, therefore the differences in their expression cannot be discussed.

Although *CD86* and *CD40* encode activation proteins, only basal levels can be evaluated in these results. I cannot discuss their role as activation markers because cells were not activated at the moment of sample preparation. *CD86* exhibited a high expression in Healthy Controls and Stable patients while Tolerant patients exhibited a median expression. Chronic Rejector exhibited the lowest *CD86* expression. *CD40* gene expression, as well as CD40 protein expression, did not exhibit any difference between patient groups. Contrary to these results, Pallier *et al*. found that tolerant patients high express CD86 and CD40 at basal levels (Pallier *et al*. 2010).

TLR5 and *TLR9* are Toll like receptors-encoding genes. Even though is difficult to evaluate differences in *TLRs* expression in non-activated cells, *TLR5* was an important gene to evaluate because was found down-regulated in Tolerant patients from the IOT study (Sagoo *et al*. 2010). Low expression of *TLR5* in chronic rejector was detected, while *TLR9* expression did not manifest any difference between the other patient groups.

Regarding proteins-encoding genes from the BCR pathway signalling, *BLNK* and *BTK* exhibited the same pattern than phenotypic proteins-encoding genes in which genes were highly express in all patient groups except chronic rejector. ERK exhibited a high expression in healthy controls, a median expression in stable patients and a poor expression in tolerant and chronic rejector. This is an interesting result because agrees with data from the flow panel, and together with *CD86* expression, both suggest that B cells from tolerant patients present a poor activation pattern at basal levels.